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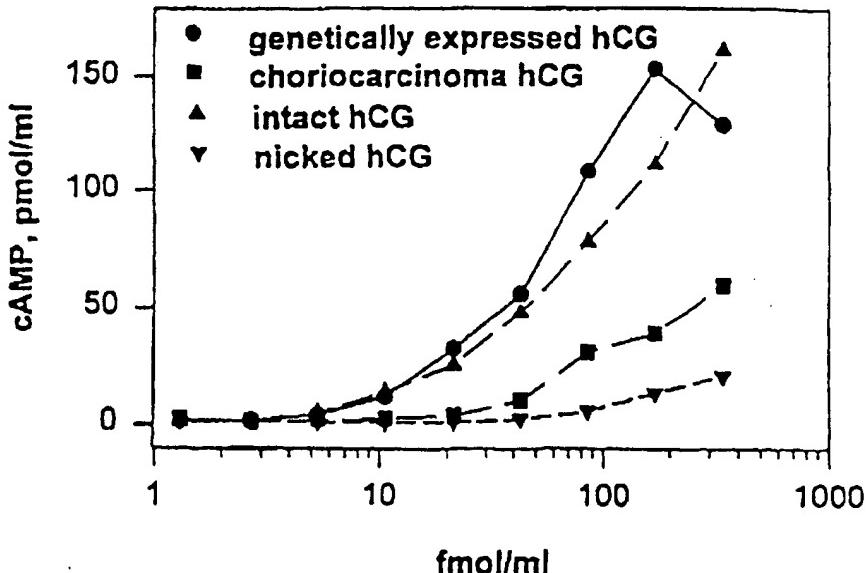
(57) Abstract

     The present invention provides a method of predicting pregnancy outcome in a subject by determining the amount of an early pregnancy associated molecular isoform of hCG in a sample. The present invention further provides a method for determining the amount of early pregnancy associated molecular isoforms of human chorionic gonadotropin (hCG) in a sample. The present invention also provides a diagnostic kit for determining the amount of early pregnancy associated hCG in a sample. The present invention additionally provides an antibody which specifically binds to an early pregnancy associated molecular isoform of human chorionic gonadotropin. Finally, the present invention provides methods for detecting trophoblast or non–trophoblast malignancy in a sample.

# METHODS FOR PREDICTING
## PREGNANCY OUTCOME IN A SUBJECT BY hCG ASSAY

5   This application claims priority of and is a continuation-in-part of U.S. Serial No. 09/311,428, filed May 13, 1999, the contents of which are hereby incorporated by reference into this application.

10

The invention disclosed herein was made with United States Government support under National Institutes of Health Grant Nos. NIEHS ES-07589 and HD 15454. Accordingly, the U.S. Government has certain rights in this invention.

15

### Background of the Invention
Throughout this application, various publications are referenced by author and date. Full citations for these publications may be found listed alphabetically at the end

20  of the specification immediately preceding the claims. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art.

25  Early pregnancy loss (EPL) is a widespread, but largely undiagnosed problem. In order to adequately diagnose and develop treatments for EPL it is essential to be able to detect and measure the rate of occurance of EPL. This is critically important in epidemiological studies, some of

30  which are related to exposures to known or suspected reproductive toxins in the workplace, in the environment or by personal use. These early pregnancy losses are often not recognized by women or physicians and are detected solely by the measurement of hCG in the urine at the time between

35  implantation and expected menses. They are sometimes termed "chemical pregnancies" or "occult pregnancies." A landmark epidemiological study established that the incidence of EPL was 22% in a population of healthy women attempting to

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conceive (Wilcox, A. J., et al., 1988). This investigation employed a very sensitive (0.01 ng/ml hCG) assay which detected only the intact hCG molecule with the unique beta subunit carboxyterminal peptide present.

5

There are multiple likely causes for EPL and clinical spontaneous abortion including genetic abnormality, immunological dysfunction, untreated infection or other unknown physiological problems. In addition, losses may be
10 caused by failure of human chorionic gonadotropin (hCG) to induce adequate response at its target, the corpus luteum. This could result from inadequate hormonal potency. "Nicking" of the beta subunit in the loop 2 region of the molecule, specifically between residues 44-49, can reduce
15 biopotency of hCG. Cleaved peptide bonds in this area of the molecule also exhibit reduced biopotency and reduced immunochemical recognition by monoclonal antibodies directed to the heterodimeric hormone (Cole, L. A., et al., 1991a; Cole , L. A., et al., 1991b; Puisieux, A., et al., 1990;
20 Nishimura, R., et al., 1988; Nishimura, R. T., et al., 1989). Nicked forms of hCG were examined as possibly more prevalent in EPL situations and, at least in part responsible, for early pregnancy loss. Unfortunately many of the reports claiming that substantial concentrations of nicked hCG are
25 produced during pregnancy, losses or successful pregnancies, are not accurate due to faulty assumptions regarding assay specificity (Wilcox, A. J., et al., 1988). Carbohydrate-modified hCG can also exhibit reduced biopotency. It is known that if the hCG has much reduced sialic acid content
30 and its carbohydrate chains terminate in galactose, much hCG would be removed by the liver receptor for such altered glycoproteins (Braun, J. R., et al., 1996; Kawasaki, T. and G. Ashwell, 1996). The circulating life-time of asialo hCG is reduced and its in vivo potency is thereby low. Other
35 carbohydrate changes also alter circulating half life;

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glycoproteins terminating in sulfate-N-acetyl galactosamine are also extracted by a specific liver receptor and have reduced circulating lifetime (Baenziger, J. U., 1994; Fiete, D., et al., 1991).

5

At least two factors affect increased potency of hCG. First, it is known that a larger Stoke's radius will decrease clearance through the kidney glomerulus which generally clears proteins above an effective size of 70,000 very

10 slowly. The effective size of urinary-isolated hCG is just at this borderline reduced clearance size. Generally, extra sugar content makes the hydrated radius of glycoproteins larger. It has been shown that by adding the hCG beta COOH-terminal peptide to hFSH or hLH, their circulating life-times

15 greatly increased (Fares, F. A. et al., 1992; Matzuk, M. M., 1990). This addition was thought mostly due to the carbohydrate content of that peptide rather than simply the extra polypeptide size (Wilcox, A. J., et al., 1988). Second, increased negative charge of a protein will prolong

20 its circulating time because of decreased renal clearance (Chmielewski, C. 1992, Quadri, K. H., et al., 1994; Maack, T., et al., 1985). This increased negative charge can be due to extra sialic acid or other negative groups, including sulfate such as is present on hLH and on the pituitary form

25 of hCG (Birken, S., et al., 1996b). Changes which affect signal transduction at the receptor may also affect biopotency of hCG. It is known that deglycosylated hCG has much reduced receptor potency (Ravindranath, N., et al., 1992; Sairam, M. R., and L. G., Jiang, 1992; Browne, E. S.,

30 et al., 1990; Sairam, M. R., 1989; Sairam, M. R., et al., 1988). Carbohydrate reduced forms of hCG also have reduced signal transduction (Amano, J., et al., 1990; Bahl, O. P., et al., 1995; Moyle, W. R., 1975).

35 According to the present invention EPL or recurrent spontaneous abortion is not due to an abnormal hCG form that

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has reduced potency, such as nicked hCG. Instead, the present invention provides evidence that in successful outcome pregnancies women usually produce forms of hCG which are very highly potent in very early pregnancy; the standard
5 urinary reference preparations of hCG are less potent forms of the hormone produced later in pregnancy. The increased potency could be caused by a combination of factors from circulating half-life to increased receptor affinity or signal transduction or all of the preceding. Since hCG is
10 low very early in pregnancy, it is logical to find a more potent form of hCG on a molar basis to carry out its function until production levels rise as the trophoblastic cellular mass increases. The present invention describes molecular and immunological tools and methods including an antibody,
15 B152, described herein which recognizes the highly potent early pregnancy associated molecular isoforms of hCG. The determination of blood and urine profiles for the B152 hCG isoforms throughout healthy pregnancies can delineate the pattern of isoforms in successful pregnancies. These
20 isoforms can be measured by immunoassay alone, obviating the need to perform complex isoelectric focusing studies or other separation techniques. Additionally, the methods described herein are applicable to large numbers of samples.

5

## Summary of the Invention

The present invention provides a method of predicting pregnancy outcome in a subject by determining the amount of an early pregnancy associated molecular isoform of hCG in a

5   sample comprising: (a) contacting a sample with an antibody which specifically binds to the early pregnancy associated molecular isoform of hCG under conditions permitting formation of a complex between the antibody and the early pregnancy associated molecular isoform of hCG; (b) measuring

10  the amount of complexes formed, thereby determining the amount of the early pregnancy associated molecular isoform of hCG in the sample; and (c) comparing the amount early pregnancy associated molecular isoform of hCG in the sample determined in step (b) with either (i) the amount determined

15  for temporally matched, normal pregnant subject(s) or (ii) the amount determined for non-pregnant subject(s), wherein the relative absence of the early pregnancy associated molecular isoform of hCG in the sample indicates a negative outcome of pregnancy for the subject.

20

The present invention further provides a method of predicting pregnancy outcome in a subject by determining the amount of an early pregnancy associated molecular isoform of hCG in a sample comprising: (a) contacting a capturing antibody which

25  specifically binds to the early pregnancy associated molecular isoform of hCG with a solid matrix under conditions permitting binding of the antibody with the solid matrix; (b) contacting the bound matrix with the sample under conditions permitting binding of the antigen present in the sample with

30  the capturing antibody; (c) separating the bound matrix and the sample; (d) contacting the separated bound matrix with a detecting antibody which specifically binds to hCG under conditions permitting binding of antibody and antigen in the sample; (e) measuring the amount of bound antibody on the

35  bound matrix, thereby determining the amount of early

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pregnancy associated molecular isoform of hCG in the sample;
and (f) comparing the amount early pregnancy associated
molecular isoform of hCG in the sample determined in step (e)
with either (i) the amount determined for temporally matched,
5   normal pregnant subject(s) or (ii) the amount determined for
non-pregnant subject(s), wherein amounts of the early
pregnancy associated molecular isoform of hCG in the sample
similar to amounts of early pregnancy associated molecular
isoform of hCG in temporally matched' pregnant samples
10  indicates a positive outcome, amounts of early pregnancy
associated molecular isoform of hCG in the sample similar to
amounts of early pregnancy associated molecular isoform of
hCG in the non-pregnant samples indicates a negative outcome
of pregnancy for the subject.
15

In addition, the present invention provides a method for
determining the amount of early pregnancy associated
molecular isoforms of in a sample comprising: (a) contacting
the sample with an antibody which specifically binds to an
20  early pregnancy associated molecular isoform of hCG under
conditions permitting formation of a complex between the
antibody and the early pregnancy associated molecular isoform
of hCG; and (b) determining the amount of complexes formed
thereby determining the amount of early pregnancy associated
25  molecular isoform of hCG in the sample.

Further, the present invention provides a diagnostic kit for
determining the amount of early pregnancy associated hCG is
a sample comprising: (a) an antibody which specifically binds
30  to an early pregnancy associated molecular isoform; (b) a
solid matrix to which the antibody is bound; and (c) reagents
permitting the formation of a complex between the antibody
and a sample.

35  The present invention additionally provides an antibody which
specifically binds to an early pregnancy associated molecular

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isoform of human chorionic gonadotropin.

Further, the present invention provides a method for
detecting non-trophoblast malignancy in a sample comprising:
5 (a) contacting a sample with an antibody which specifically
binds to the early pregnancy associated molecular isoform
of hCG under conditions permitting formation of a complex
between the antibody and the early pregnancy associated
molecular isoform of hCG; (b) contacting the sample with a
10 second detection antibody which specifically binds to intact
non-nicked hCG without substantially cross-reacting with
said antibody under conditions permitting formation of a
complex between the antibody and the early pregnancy
associated molecular isoform of hCG; (c) measuring the amount
15 of complexes formed, thereby determining the amount of the
early pregnancy associated molecular isoform of hCG in the
sample; and (d) comparing the amount of early pregnancy
associated molecular isoform of hCG in the sample determined
in step (b) with the amount of early pregnancy associated
20 molecular isoform of hCG in the sample determined in step
(c), wherein a positive detection of early pregnancy
associated molecular isoform detected in step (b) and a
relative absence of the early pregnancy associated molecular
isoform of hCG detected in step (c) indicates the presence
25 of non-trophoblast malignancy in the sample.

Finally, the present invention provides a method for
detecting gestational trophoblast disease in a sample from
a subject comprising (a) contacting a sample with an antibody
30 which specifically binds to the early pregnancy associated
molecular isoform of hCG under conditions permitting
formation of a complex between the antibody and the early
pregnancy associated molecular isoform of hCG; (b) contacting
the sample with a second detection antibody which
35 specifically binds to intact non-nicked hCG without
substantially cross-reacting with said antibody under

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conditions permitting formation of a complex between the antibody and the early pregnancy associated molecular isoform of hCG; (c) measuring the amount of complexes formed, thereby determining the amount of the early pregnancy associated
5   molecular isoform of hCG in the sample due to binding with the first antibody, and late pregnancy associated molecular isoform of hCG in the sample due to binding with the second antibody; (d) determining the ratio of early pregnancy associated molecular isoform of hCG to late pregnancy
10  associated molecular isoform of hCG in the subject; and (e) comparing the ratio of early pregnancy associated molecular isoform of hCG to late pregnancy associated molecular isoform of hCG in the sample determined in step (c) over time, wherein a continuing high ratio of early pregnancy associated
15  molecular isoform of hCG to late pregnancy associated molecular isoform of hCG in the sample determined in step (c) indicates the presence of gestational trophoblast disease in the subject.

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## Brief Description of the Figures

### Figure 1

Bioassay for forms of hCG.  This is data from recombinant CHO cells expressing the LH/CG receptor. The response factor is cAMP production.  The x-axis is dose of one of four calibrated, pure hormones as described on graph legends.  Expressed hCG has no nicks; choriocarcinoma hCG (C5) is 100% nicked; CR 127 was purified into a nick-free (non-nicked, intact) and nick-enriched fraction as shown.

### Figure 2

Incidence (Panel A) and expression level (Panel B) of hCG-related molecules in the positive samples for each of the analyses measured (In early normal pregnancy, n=214; EPL cycles, n=49; and negative cycles, n=297).

### Figure 3

Binding curves for three hCG types in the B152-B207* assay (upper panel) and the B109-B108* assay (lower panel).

### Figures 4

Ratio of hCG isoforms measured by the B152-B207* and B109-B108* assays in normal pregnancy urine (n=103) at different gestational ages.  (Regression curve and 95% confidence intervals are shown, $r^2=0.79$).  An inflection point in the curve occurs at approximately 29 weeks.

### Figures 5

Box plot of the B152/B109 ratio for pregnancy matched serum/urine at 5-6 weeks of gestational age (n=12); or at 36-39 weeks of gestational age (n=11) and in JAR cell supernatant.  Box extends to the 25th and 75th percentile.  The upper and lower symbols indicate the

10

90th and 10th percentile respectively. A solid line
inside the box marks the value of the 50th percentile.


**Figure 6**

5       Ratio of hCG isoforms measured by the B152-B207* and
        B109-B108* in urine of IVF patients (n=65).
        (Regression curve and 95% confidence intervals are
        shown, $r^2=0.59$).


10 **Figure 7**

        Immunoassay profiles of fractions from Superose 12
        column chromatography of a pooled urine concentrate
        from pregnant women.


15 **Figure 8**

        Liquid phase radioimmunoassays using antibody B151
        (panel A) and antibody B152 (panel B). Radiolabeled
        choriocarcinoma hCG was used as tracer and calibrated
        (by amino acid analysis) solutions of pregnancy C5
20      chorioCG, hCG CR 127, hCG CR 127 non-nicked, and hCGn
        CR 127 were employed as competitors. Non-linear
        regression lines were plotted in logit transformed
        format.


25

    **Figure 9**

        Radio-Immunometric (two-site) assay using antibody B152
        as capture and B207 as radiolabeled detection reagent.
        Binding curves are shown for competitors, various
30      competitor as detailed in methods and results. Each
        panel represents a separate assay in which all ligands
        were introduced in the same assay. Points were
        connected by straight lines although regression
        analysis (4 parameter logistic) indicated excellent fit
35      to logistic or sigmoidal curve shape model. Panel A

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and B represent two distinct assays with similar
results. It is clear that this assay has greatest
recognition of the nicked, choriocarcinoma hCG
immunogen which is hyperglycosylated and binds
5    similarly to nicked and non-nicked forms of hCG which
contain the usual quantities of sugars. Reagent M1A is
missing most of its beta COOH-terminal region,
supporting a role of this region in the binding site of
B152 (see panel B and discussion in text).

10

**Figure 10**

Enzymic-Immunometric (two-site) assay using antibody
B152 as capture and peroxidase-labeled B4001 as
detection antibody. A linear-linear plot of molar
15   quantities of ligand added is plotted versus absorbance
at 492nm which is the response factor from the
peroxidase detection system. Eight different hormone
forms were measured as ligands within the same assay as
indicated in the legend. The two hyperglycosylated
20   choriocarcinoma-derived hCG isoforms are both the most
potent ligands (Figure 14). Potency correlated well
with hyperglycosylation of ligand (see Figure 10 and
text).


25 **Figure 11**

Characteristics of the Reagents Used to Define Antibody
Specificity. The peptide and carbohydrate structures
of the reagents used were determined earlier (26). The
% nicked β-subunit refers to the proportion of
30   molecules with cleavages (missing peptide bonds) in the
region β43 to β48. The % tetrasaccharide core is the
proportion of O-linked oligosaccharides with
tetrasaccharide (vs. disaccharide) core structure, and
the % sialic acid, is the proportion of O-linked
35   structures with antennae terminated by sialic acid

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residues.    The proportion of triantennary N-linked oligosaccharides on β-subunit is given, as is the corresponding % sialic acid.

[a].% sialic acid residues per sugar chain, N-linked on β.
[b].% sialic acid residues per sugar chain, O-linked on β.
[c] The "CR" series of hCG reference preparations were made at Columbia University and were distributed internationally as reference materials for purified hCG.   CR 119 is also known as the 3rd international immunoassay reference preparation for hCG.
[d] ND is not done; NA is not applicable to that reagent.
[e] Less than 15% of the beta COOH-terminal region is present on this preparation.

**Figure 12**

Affinity Constants[a] Determined by Liquid Phase Competition Assays Using C5 as Tracer Ligand.
[a]Ka as L/M
[b]hCG CR 127 is an NIH-distributed hCG reference preparation produced at Columbia University.

**Figure 13**

Matrices of data for binding characteristics of different pairs of detection antibodies using B151 or B152 as capture antibody.

A. Relative Cross-Reactivities of Two Site Assay Using B151 as Capture Antibody
[a] labeled detection antibodies
[b] out of low range detection
[c] this particular assay format was applied in O'Connor et al (25).

13

B. Relative Cross-Reactivities of Two Site Assay Using
B152 as Capture Antibody

The molar quantity of ligand required to produce
5    binding equal to 50% of the maximum binding achieved by
C5 was determined.  Cross-reactivity shown in this
figure as a percentage is calculated by dividing the
molar quantity of the standard by the molar quantity of
the other ligand at 50% maximum binding dose.
10   ª labeled detection antibodies
     ᵇ maximum binding represents the total quantity of
radiolabeled detection antibody which can bind to the
plate in the system described.
     ᶜ out of low range detection
15   ᵈ this particular assay format was applied in O'Connor
et al (25).


**Figure 14**

Immunoreactivity    of    antigens    in    the    B152
20   immunoradiometric assay.  The dose-response curves used
to provide data for this figure are shown in Figure 9.
Each curve was fitted with 4-5 points.  Slope and
coefficient of determination ($R^2$) were determined using
a non-linear regression algorithm.  Slopes were used as
25   an indicator of antigen potency.  Relative potency was
estimated as the slope of antigens relative to the
slope of C5 Choriocarcarcinoma hCG (the immunogen).

     ª Slope are from Figure 9 as calculated in Sigmaplot
30   4.01 by linear regression analysis.  Units of slope are
pmole/ml absorbance at 492nm.


**Figure 15(A-D)**

Urinary isoform profiles and ratio in normal singleton
35   pregnancies.  ET day is a day from embryo transfer.

14

**Figure 16(A-C)**

Urinary isoform profiles and ratio in normal multiple
birth pregnancy. ET day is a day from embryo transfer.


5 **Figure 17(A-E)**

Urinary isoform profiles and ratio in early pregnancy
loss. ET day is a day from embryo transfer.

15

## Detailed Description of the Invention

A method of predicting pregnancy outcome in a subject by determining the amount of an early pregnancy associated molecular isoform of hCG in a sample comprising: (a)
5 contacting a sample with an antibody which specifically binds to the early pregnancy associated molecular isoform of hCG under conditions permitting formation of a complex between the antibody and the early pregnancy associated molecular isoform of hCG; (b) measuring the amount of complexes formed,
10 thereby determining the amount of the early pregnancy associated molecular isoform of hCG in the sample; and (c) comparing the amount early pregnancy associated molecular isoform of hCG in the sample determined in step (b) with either (i) the amount determined for temporally matched,
15 normal pregnant subject(s) or (ii) the amount determined for non-pregnant subject(s), wherein the relative absence of the early pregnancy associated molecular isoform of hCG in the sample indicates a negative outcome of pregnancy for the subject.  In an embodiment of the present invention, the
20 antibody is B152.  Another embodiment of this invention is the early pregnancy associated molecular isoform of Hcg.

The hybridoma producing the B152 monoclonal antibody was deposited on February 3, 1998 with the American Type Culture
25 Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A. under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganism for the Purposes of Patent Procedure.  The hybridoma, was accorded ATCC Accession Number HB-12467.
30

According to one embodiment of this invention, step (a) further comprises a second antibody which specifically binds to hCG without substantially cross-reacting with said antibody under conditions permitting formation of a complex
35 between the antibody and the early pregnancy associated

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molecular isoform of hCG. In an embodiment of this invention, the second antibody is B207. According to another embodiment of this invention, step (a) further comprises a second antibody which specifically binds to intact non-nicked
5 hCG without substantially cross-reacting with said antibody under conditions permitting formation of a complex between the antibody and the early pregnancy associated molecular isoform of hCG. In an embodiment of this invention, the second antibody is B108 or B109. In an embodiment of this
10 invention, step (c) comprises comparing the amount of the early pregnancy associated molecular isoform of hCG determined in step (b) for said antibody with the amount determined in step (b) for the second antibody, wherein a high ratio of amounts determined for said antibody relative
15 to the second antibody indicates a positive outcome of pregnancy for the subject, a low ratio indicates a negative outcome of pregnancy for the subject.

In yet another embodiment of this invention, step (c)
20 comprises comparing the amount early pregnancy associated molecular isoform of hCG in the sample determined in step (b) with either (i) the amount determined for temporally matched, normal pregnant subject(s) or (ii) the amount determined for non-pregnant subject(s), wherein amounts of the early
25 pregnancy associated molecular isoform of hCG in the sample similar to amounts of early pregnancy associated molecular isoform of hCG in temporally matched pregnant samples indicates a positive outcome, amounts of early pregnancy associated molecular isoform of hCG in the sample similar to
30 amounts of early pregnancy associated molecular isoform of hCG in the non-pregnant samples indicates a negative outcome of pregnancy for the subject.

This invention also provides a method of predicting the
35 liklihood of a negative pregnancy outcome in a female subject comprising: (a) contacting a sample from the subject with a

capture antibody which specifically binds to an early
pregnancy associated molecular isoform of hCG under
conditions permitting formation of a complex between the
antibody and the early pregnancy associated molecular isoform
5  of hCG; (b) contacting any complex formed in step (a) with
a labelled detection antibody under conditions permitting
binding to the complex the capture antibody and the hCG
isoform; (c) measuring the amount of labeled detection
antibody bound to the complex so as to thereby determine the
10  amount of the early pregnancy associated molecular isoform
of hCG in the sample; and (d) comparing the amount early
pregnancy associated molecular isoform of hCG in the sample
determined in step (b) with the amount determined for a
normal pregnant subject, wherein the relative absence of the
15  early pregnancy associated molecular isoform of hCG in the
sample indicates a negative outcome of pregnancy for the
subject.

According to an embodiment of this invention, the sample is
20  a urinary sample or a blood sample.  In one embodiment of
this invention, the sample is an aggregate sample taken from
at least two consecutive days.  In an embodiment of this
invention, the sample is a spot urine sample, a first morning
void urine sample, or an aggregate sample of the first
25  morning void urine samples for at least two consecutive days.
In one embodiment of this invention, the antibody is labeled
with a detectable marker.  In an embodiment of this
invention, the detectable marker is a radioactive isotope,
enzyme, dye, magnetic bead, or biotin.  In a preferred
30  embodiment, the radioactive isotope is $I^{125}$.

The present invention further provides a method of predicting
pregnancy outcome in a subject by determining the amount of
an early pregnancy associated molecular isoform of hCG in a
35  sample comprising: (a) contacting a capturing antibody which
specifically binds to the early pregnancy associated

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molecular isoform of hCG with a solid matrix under conditions
permitting binding of the antibody with the solid matrix; (b)
contacting the bound matrix with the sample under conditions
permitting binding of the antigen present in the sample with
5  the capturing antibody; (c) separating the bound matrix and
the sample; (d) contacting the separated bound matrix with
a detecting antibody which specifically binds to hCG under
conditions permitting binding of antibody and antigen in the
sample; (e) measuring the amount of bound antibody on the
10  bound matrix, thereby determining the amount of early
pregnancy associated molecular isoform of hCG in the sample;
and (f) comparing the amount early pregnancy associated
molecular isoform of hCG in the sample determined in step (e)
with either (i) the amount determined for temporally matched,
15  normal pregnant subject(s) or (ii) the amount determined for
non-pregnant subject(s), wherein amounts of the early
pregnancy associated molecular isoform of hCG in the sample
similar to amounts of early pregnancy associated molecular
isoform of hCG in temporally matched pregnant samples
20  indicates a positive outcome, amounts of early pregnancy
associated molecular isoform of hCG in the sample similar to
amounts of early pregnancy associated molecular isoform of
hCG in the non-pregnant samples indicates a negative outcome
of pregnancy for the subject.
25

An embodiment of this invention further comprises (a)
removing of the sample from the matrix; and (b) washing the
bound matrix with an appropriate buffer. In one embodiment
of this invention, the capturing antibody is B152. In one
30  embodiment of this invention, the detecting antibody is B207.
In an embodiment of this invention, step (a) further comprises
a second capturing antibody which specifically binds to
intact non-nicked hCG without substantially cross-reacting
with said antibody under conditions permitting formation of
35  a complex between the antibody and the early pregnancy
associated molecular isoform of hCG. According to an

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embodiment of this invention, the second capturing antibody
is B108 or B109.  In an embodiment of this invention, step
(d) further comprises a second detecting antibody which
specifically binds to hCG without substantially cross-
5 reacting with said antibody under conditions permitting
formation of a complex between the antibody and the early
pregnancy associated molecular isoform of hCG.  In an
embodiment of this invention, step (f) comprises comparing
the amount of the early pregnancy associated molecular
10 isoform of hCG determined in step (e) for said antibody with
the amount determined in step (b) for the second antibody,
wherein a high ratio of amounts determined for said antibody
relative to the second antibody indicates a positive outcome
of pregnancy for the subject, a low ratio indicates a
15 negative outcome of pregnancy for the subject.

According to an embodiment of this invention, the sample is
a urinary sample or a blood sample.  In one embodiment of
this invention, the sample is an aggregate sample taken from
20 at least two consecutive days.  In an embodiment of this
invention, the sample is a spot urine sample, a first morning
void urine sample, or an aggregate sample of the first
morning void urine samples for at least two consecutive days.
In one embodiment of this invention, the antibody is labeled
25 with a detectable marker.  In an embodiment of this
invention, the detectable marker is a radioactive isotope,
enzyme, dye, magnetic bead, or biotin.  In a preferred
embodiment, the radioactive isotope is $I^{125}$.

30 In addition, the present invention provides a method for
determining the amount of early pregnancy associated
molecular isoforms of in a sample comprising: (a) contacting
the sample with an antibody which specifically binds to an
early pregnancy associated molecular isoform of hCG under
35 conditions permitting formation of a complex between the
antibody and the early pregnancy associated molecular isoform

of hCG; and (b) determining the amount of complexes formed thereby determining the amount of early pregnancy associated molecular isoform of hCG in the sample.

5 According to an embodiment of this invention, the antibody specifically binds a region of the early pregnancy associated molecular isoform of hCG comprising a carbohydrate moiety. In one embodiment of this invention the antibody is produced by a hybridoma cell line. In one embodiment of this
10 invention the antibody is B152. In another embodiment the antibody specifically binds to the nicked form of human gonadotropin. The antibody is unlabeled or labeled with a detectable marker. In one embodiment of the invention the detectable marker is a detection antibody. In another
15 embodiment the antibody specifically binds an epitope dependent upon peptide bond cleavage in beta loop 2. In still another embodiment the antibody binds the human gonadotropin at the beta COOH-terminal region.

20 Further, the present invention provides a diagnostic kit for determining the amount of early pregnancy associated hCG is a sample comprising: (a) an antibody which specifically binds to an early pregnancy associated molecular isoform; (b) a solid matrix to which the antibody is bound; and (c) reagents
25 permitting the formation of a complex between the antibody and a sample. In an embodiment of this invention, the antibody is B108, B109 or B152. An embodiment of this invention further comprises control sample(s) normal pregnant sample(s), nonpregnant sample(s), or male sample(s).
30

According to an embodiment of this invention, the sample is a urinary sample or a blood sample. In one embodiment of this invention, the sample is an aggregate sample taken from at least two consecutive days. In an embodiment of this
35 invention, the sample is a spot urine sample, a first morning void urine sample, or an aggregate sample of the first

morning void urine samples for at least two consecutive days.
In one embodiment of this invention, the antibody is labeled
with a detectable marker. In an embodiment of this
invention, the detectable marker is a radioactive isotope,
5 enzyme, dye, magnetic bead, or biotin. In a preferred
embodiment, the radioactive isotope is I$^{...}$.

The present invention additionally provides an antibody which
specifically binds to an early pregnancy associated molecular
10 isoform of human chorionic gonadotropin.

In an embodiment of this invention, the antibody specifically
binds to a region of the early pregnancy associated molecular
isoform of human chorionic gonadotropin comprising a
15 carbohydrate moiety. According to one embodiment of this
invention, the monoclonal antibody is B152. In an embodiment
of this invention, a hybridoma cell (ATCC Accession No.HB-
12467 ) is provided capable of producing monoclonal antibody
B152. Another embodiment of this invention is the early
20 pregnancy associated molecular isoform of hCG recognized by
the B152 monoclonal antibody.

Further, the present invention provides a method for
detecting non-trophoblast malignancy in a sample comprising:
25 (a) contacting a sample with an antibody which specifically
binds to the early pregnancy associated molecular isoform
of hCG under conditions permitting formation of a complex
between the antibody and the early pregnancy associated
molecular isoform of hCG; (b) contacting the sample with a
30 second detection antibody which specifically binds to intact
non-nicked hCG without substantially cross-reacting with
said antibody under conditions permitting formation of a
complex between the antibody and the early pregnancy
associated molecular isoform of hCG; (c) measuring the amount
35 of complexes formed, thereby determining the amount of the
early pregnancy associated molecular isoform of hCG in the

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sample; and (d) comparing the amount of early pregnancy
associated molecular isoform of hCG in the sample determined
in step (b) with the amount of early pregnancy associated
molecular isoform of hCG in the sample determined in step
5 (c), wherein a positive detection of early pregnancy
associated molecular isoform detected in step (b) and a
relative absence of the early pregnancy associated molecular
isoform of hCG detected in step (c) indicates the presence
of non-trophoblast malignancy in the sample.
10

According to an embodiment of this invention, the antibody
is B152 or B109.  In an embodiment of this invention, the
detection antibody is B207 for  B152 assay, B108 for B109
assay.  In an embodiment of this invention, the non-
15 trophoblast malignancy is ovarian malignancy or prostate
malignancy.


According to an embodiment of this invention, the sample is
a urinary sample or a blood sample.  In one embodiment of
20 this invention, the sample is an aggregate sample taken from
at least two consecutive days.  In an embodiment of this
invention, the sample is a spot urine sample, a first morning
void urine sample, or an aggregate sample of the first
morning void urine samples for at least two consecutive days.
25 In one embodiment of this invention, the antibody is labeled
with a detectable marker.  In an embodiment of this
invention, the detectable marker is a radioactive isotope,
enzyme, dye, magnetic bead, or biotin.  In a preferred
embodiment, the radioactive isotope is $I^{125}$.
30

In an embodiment, the present invention provides a method for
detecting gestational trophoblast disease in a sample from
a subject comprising (a) contacting a sample with an antibody
which specifically binds to the early pregnancy associated
35 molecular isoform of hCG under conditions permitting
formation of a complex between the antibody and the early

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pregnancy associated molecular isoform of hCG; (b) contacting the sample with a second detection antibody which specifically binds to intact non-nicked hCG without substantially cross-reacting with said antibody under
5 conditions permitting formation of a complex between the antibody and the early pregnancy associated molecular isoform of hCG; (c) measuring the amount of complexes formed, thereby determining the amount of the early pregnancy associated molecular isoform of hCG in the sample due to binding with
10 the first antibody, and late pregnancy associated molecular isoform of hCG in the sample due to binding with the second antibody; (d) determining the ratio of early pregnancy associated molecular isoform of hCG to late pregnancy associated molecular isoform of hCG in the subject; and (e)
15 comparing the ratio of early pregnancy associated molecular isoform of hCG to late pregnancy associated molecular isoform of hCG in the sample determined in step (c) over time, wherein a continuing high ratio of early pregnancy associated molecular isoform of hCG to late pregnancy associated
20 molecular isoform of hCG in the sample determined in step (c) indicates the presence of gestational trophoblast disease in the subject.

In an embodiment of this invention, the antibody is B152 or
25 B109. In another embodiment of this invention, the second antibody is B108 for B109 B207 for B152 assay. In an embodiment of the present invention, the gestational trophoblast disease is choriocarcinoma or hydatidiform mole.

30 According to an embodiment of this invention, the sample is a urinary sample or a blood sample. In one embodiment of this invention, the sample is an aggregate sample taken from at least two consecutive days. In an embodiment of this invention, the sample is a spot urine sample, a first morning
35 void urine sample, or an aggregate sample of the first morning void urine samples for at least two consecutive days.

24

In one embodiment of this invention, the antibody is labeled
with a detectable marker.   In an embodiment of this
invention, the detectable marker is a radioactive isotope,
enzyme, dye, magnetic bead, or biotin.   In a preferred
5 embodiment, the radioactive isotope is I.

As described herein below, unexpected isoforms of hCG are
produced during normal early pregnancy.  Using an *in vitro*
bioassay, it appears that these isoforms have enhanced
10 potency for signal transduction.   These isoforms can be
measured using the novel sensitive, immunoassay described
herein.  This can help predict pregnancy outcome where one
cause of early pregnancy loss is failure to produce the
isoform of hCG of higher potency produced by successful
15 pregnancies.  This enables physicians to intervene to sustain
a failing pregnancy.  Identification of the nature of the hCG
isoform required might provide the proper reagent needed to
sustain pregnancy.

20 New antibodies for measurement of nicked forms of hCG
described herein below were developed based on the hypothesis
that forms of hCG, which have greatly reduced bioactivity,
contribute to early pregnancy loss (EPL), due at least in
part to diminished biopotency.  Evidence was found that the
25 hCG that appears in EPL patients displays reduced biological
activity. However, it was determined that the cause of the
reduced bioactivity is not the presence of nicked hCG in EPL
patients.   Instead, the hypothesis is that patients that
carry pregnancies forward produce an isoform of hCG with
30 enhanced bioactivity.   The instant invention describes a
unique immunochemical assay to measure this unexpected and
previously uncharacterized isoform of early pregnancy hCG
directly in clinical samples of blood and urine. One of the
antibodies developed reacted against a nicked form of hCG
35 isolated from a choriocarcinoma patient, was not specific for

a nicked form of hCG but appeared to discriminate among
carbohydrate variants of hCG.  This antibody, designated
B152, appears to preferentially bind hCG forms from
choriocarcinoma patients.  In studying the content of hCG
5  isoforms during pregnancy, the unique and unexpected
observation was made that B152 in the first four weeks of
pregnancy measured much higher quantities of an isoform of
hCG as compared to the standard hCG isoforms measured by the
usual heterodimeric hCG assays exemplified by a previously
10  described B109 based assay.  In fact, in early pregnancy
(days 9,10,11 postovulation) B152 measured as much as 20-fold
more hCG, than did another monoclonal antibody, B109.  Later
in pregnancy, the B152 isoform declines and is lower in third
trimester pregnancy urine than the standard isoforms measured
15  by B109.  A further striking observation was that in very
early pregnancy, a high B152/B109 ratio correlates with a
successful pregnancy outcome while a low ratio correlated
with pregnancy loss.  This discovery is important as the
potentially overlooked isoforms of hCG described herein
20  during pregnancy may be predictors of successful pregnancy
outcome.  Such an assay has wide medical applications and
provides a clinician with opportunity to intervene very early
in pregnancy if the assay indicated that the pregnancy
appeared troubled.

25

An antibody, designated B152, produced by the hybridoma cell
accorded ATCC Accession number HB-12467, generated against
a nicked form of hCG isolated from a choriocarcinoma patient,
but not specific for nicked isoform hCG is able to
30  discriminate among carbohydrate variants of hCG.  B152 is
specific for an early pregnancy associated molecular isoform
of hCG, which in the first four weeks of pregnancy is
measured at much higher quantities than the hCG standard
isoforms measured by the usual heterodimeric hCG assays
35  exemplified by a previously described B109 based assay.
Later in pregnancy, the B152 isoform declines and is lower

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in third trimester pregnancy urine than the standard isoforms
measured by 3109.


This invention is illustrated in the Experimental Details
5 section which follows.  These sections are set forth to aid
in an understanding of the invention but are not intended to,
and should not be construed to, limit in any way the
invention as set forth in the claims which follow thereafter.

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## EXPERIMENTAL DETAILS FOR THE FIRST SERIES OF EXPERIMENTS

Example 1:     Antibodies to and Analysis of Molecular
               Isoforms of hCG in Early Pregnancy and Early
5              Pregnancy Loss

### Introduction

Almost all investigations of the incidence of early pregnancy
loss (EPL), either in normal populations or in populations
10 at risk as a consequence of exposure to putative reproductive
toxins (Hakim, R. B., et al., 1995; Lasley, B. L., et al.,
1995) use assays for heterodimeric, non-nicked hCG or
combination assays which include free beta subunit and beta
core fragment of hCG. One concern about the forms of hCG to
15 include in the measurement in EPL was heightened with respect
to the nicking phenomenon described above. Because nicked
hCG molecules are not measured by the antibodies employed
in most EPL studies, the incidence of EPL is presumably
underestimated by an amount proportional to the extent of
20 nicking in the urinary molecule. Another concern of
significant importance was a determination of the nature of
the "hCG like" immunoreactivity in the urine in the
periovulatory surge of the menstrual cycle (O'Connor J., et
al., 1995). Recent reports have confirmed the existence of
25 and documented the structure of a sulfated form of hCG
produced in the pituitary (Birken, S., et al., 1996b). There
is a pulsatile secretion of hCG in both men and non-pregnant
women. (Odell, W. D.; Griffin, J., 1989 and Odell, W. D.;
Griffin, J., 1987). The presence of a non-pregnancy
30 associated form of sulfated hCG of pituitary origin, peaking
at ovulation and perhaps persisting into the luteal phase,
could potentially interfere with the accurate estimation of
EPL.

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Unappreciated isoforms of hCG in blood and urine very early
in pregnancy may be more potent *in vivo* than the forms of hCG
produced later in pregnancy. The absence of such isoforms
may be one cause of early pregnancy loss. A sensitive and
5  specific immunoassay system was designed and made to measure
unique early pregnancy associated molecular isoforms (EPMI)
of hCG. These isoforms, likely to differ by carbohydrate
composition, are predictive of a successful pregnancy
outcome. When these early pregnancy associated molecular
10 isoforms of hCG are absent or present in low concentration,
the pregnancy may be lost very early and be observed as only
a "chemical" pregnancy. These hCG isoforms may resemble the
forms of hCG produced in some choriocarcinoma patients from
which the immunogen used to produce monoclonal antibody B152
15 was derived as described herein below. The isoforms resemble
those from trophoblastic disease not in terms of nicking or
intact peptide chains but likely in carbohydrate content.
The present invention describes that the molar ratio of B152
to B109 epitopes are predictive of a successful pregnancy or
20 a loss. Three categories of pregnant patients were analyzed:
(a) normal pregnant women, (b) women who experience recurrent
abortions, (c) women undergoing embryo implantation.

It is possible to determine the hCG isoforms present in the
25 blood and urine of women who have a history of recurrent
spontaneous abortion and a similar analysis of women
undergoing embryo implantation. The combined EPL and
spontaneous abortion rate in healthy populations is 31%.
Subjects who experience three consecutive recurrent
30 spontaneous abortions have a 32% risk of sustaining another
(Hill, J. A.; Anderson, D. J., 1990). In *in vitro*
fertilization IVF pregnancy, the loss rate is 70% with non-
donor sperm and 50% when donor sperm is used. Delineation
of pregnancies with a negative outcome from pregnancies with
35 a positive outcome can be based on differences in the

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concentrations of EPMI hCG isoforms (i.e. as differences in the B152/B109 ratio in patients). In addition, specimens from gestational trophoblastic disease (GTD) can be used to discriminate between GTD and normal pregnancy.

5

## Results

### *In vitro bioassay for hLH/hCG*

An hCG bioassay was constructed employing CHO cells expressing functional human LH/CG receptor. Table 1

10 illustrates the differences *in vitro* in biological activity between nicked and non-nicked hCG as measured by this assay. This system, has been used to evaluate the activity of pituitary and placental hCG (Birken, S., et al., 1996b). Preparations of hCG were tested for nicked and non-nicked

15 molecular isoforms of hCG in a second recombinant bioassay system (Ho, H-H., et al., 1997). Similar results were obtained in both systems.

Normal pregnancy values compared with EPL values. Results

20 indicated that nicked hCG is not a significant molar constituent of either early pregnancy or EPL. Data indicated that biological activity is not correlated with nicked hCG, but is instead ascribed to a form of hCG recognized by the B152 monoclonal antibody ATCC # HB-12467 an early pregnancy

25 associated molecular isoform of hCG (EPMI hCG). It has been established that there is diminished hCG bioactivity associated with EPL as compared to early normal pregnancy (Ho, H-H., et al., 1997). Thus, diminished hCG biological activity is a factor in EPL as a consequence of a heretofore

30 unappreciated isoform of hCG – an early pregnancy associated molecular isoform of hCG.

hCG Urinary Analytes. Metabolites of hCG and hLH were studied in a variety of states (Birken, S., et al., 1996a).

35 One study indicated a 31% pregnancy loss (Zinaman, MJ, et

30

al., 1996) while another indicated a 17.4% rate of early
pregnancy loss based on hCG assays (Ellish, N. J., et al.,
1996). It is known that hCG and hCG beta core can be readily
transferred from the uterus to the circulation even in the
5 absence of implantation (Chang, P. L., 1997). The molecular
spectrum of hCG urinary analytes in EPL cycles, normal
conceptive cycles and non-conceptive cycles has been
evaluated.   The study design and demographics of the
investigation have been described (Ellish, N. J., et al.,
10 1996).

Briefly, three urine specimens per cycle, corresponding to
days 9,10, 11, post calculated day of ovulation were
collected and analyzed in a screening assay (the "combo")
15 which simultaneously detects intact, non-nicked hCG, hCG free
beta subunit, and hCG beta core fragment.   Individual
determinations for each of these analytes, as well as for
nicked hCG, and the form of intact hCG detected by monoclonal
antibody B152 (EPMI hCG) were performed on these specimens.
20 In addition, since the concentration of luteal phase hLH
urinary analytes is a concern because of cross-reaction in
hCG assays, levels of intact hLH, hLH free beta subunit and
hLH beta core fragment were determined in the normal
pregnancy cycles and the non-conceptive cycles.   Table 1
25 summarizes the characteristics of immunometric assays
employed.

30

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**TABLE 1.** Assay format and specificity

| Assay format | Primary analyte | % cross-reactivity with related analytes |
|---|---|---|
| 5 | B109-B108* | intact non-nicked hCG | <1%[b] |
| B201-C104* | hCG free beta subunit (non-nicked+nicked) | 1% hCG; 10% hCG nicked (pregnancy); <1%[b] |
| 10 | B210-B108* | hCG beta core fragment | 2% hLH beta core fragment; <1%[b] |
| B151-B207* | hCG nicked | 10% hCG nicked free beta subunit; 12% hCG non-nicked; 2% hCG free beta subunit; 2% hLH; 5% hLH free beta subunit; <1%[b] |
| B152-B207* | choriocarcinoma hCG (C5) and choriocarcinoma hCG free beta subunit | 100% hCG nicked (C5); 190% hCG free beta nicked (from C5); 10% hCG nicked (pregnancy); 5% hCG free beta nicked (pregnancy); 7% hCG (pregnancy); 6% hCG free beta subunit; <1%[b] |

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| B406- A201* | hLH | <1%ᵃ |
|---|---|---|
| B505- B503* | hLH beta core fragment | <1%ᵃ |
| B408- B409* | hLH free beta subunit | 29% hLH; <1%ᵃ |

ᵃ-(if not indicated) hLH, free beta hLH, hLH beta core fragment, hCG, free beta hCG, hCG beta core fragment;

10 ᵇ-(if not indicated ) the same as ᵃ plus nicked hCG and nicked free beta hCG (pregnancy).

The results indicate that nicked hCG does not constitute a significant mole fraction of urinary hCG immunoreactivity in
15 either EPL or early normal pregnancy. In addition, there is a substantial excretion of hCG free beta subunit in some subjects in both pregnancy and EPL. Further, both EPL and normal pregnancy cycles variably express all of the measured analytes. Although both the incidence and level of
20 expression are different between EPL's and normal pregnancy, there is no hCG related analyte unique to either state. There was, however, a clear difference between the hLH associated analytes in the control population (non-conceptive cycles) and the normal pregnancy group. Virtually all of the
25 non-pregnancy cycles expressed hLH free beta subunit and hLH beta core fragment while only a third of the conceptive cycles had detectable levels of either analyte. Intact hLH proved to be a minor constituent of the hLH profile in both groups.
30

These findings demonstrate both the necessity of measuring hCG beta core fragment in the detection of EPL, and also of making sure that the hCG beta core assay does not cross-react with beta core hLH, which is demonstrated to be present
35 in that part of the luteal phase where EPL measurements are performed. The data is summarized in Figure 2.

Statistical analysis was performed after transformation of analyte values to mole fractions so as to produce a more useful analysis due to the wide excursion of hCG analyte values among groups. The mole fraction data were evaluated

5 by discriminant analysis and by a mixed effects model incorporating LMP (last menstrual period date). The discriminant analysis was performed both with and without "outliers" (defined as values greater than two standard deviation from the mean) removed. Both approaches produced

10 similar results.

A quadratic discriminant analysis based on a cross-validation method in order to minimize bias correctly classified 91% of the normal pregnancy subjects and 80% of the EPL subjects.

15
The mixed effects analysis, testing for interactions between mole fraction of analyte and time since LMP found no significant time or group (EPL vs. normal) effects in the intact hCG assay. In the free beta subunit of hCG assay,

20 there is a significant group effect but no time trend. In both the hCG beta core fragment measurement and the B152 measurement, both the hormone levels and the time trend from LMP were significantly different between the EPL and pregnancy groups. This study produced several important

25 findings. It defined the spectrum of analytes which in both early pregnancy and EPL, thereby resolving the issue of which hCG analytes to measure in epidemiological studies in which EPL is the end point determination. More importantly, it illustrated for the first time that there are significant

30 differences both in the pattern of analytes and the time course of their appearance between early normal pregnancy and EPL. This observation facilitates very early prediction of a distressed pregnancy by urinary hCG measurements at a time which would permit therapeutic intervention.

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Immunoreactivity of different forms of hCG in the two IRMA's
(B152-B207 and B109-B108)

The relative binding of three different forms of hCG (urinary
5  hCG, pituitary hCG and choriocarcinoma hCG C5) has been
characterized in the two hCG assays (Figure 3). Urinary non-
nicked hCG and pituitary hCG are recognized nearly equally
well by the two IRMASs, while C5 recognition is quite
different. The B152-B207* assay is more sensitive to C5,
10  which is to be expected because B152 antibody was developed
and selected on the basis of higher affinity to C5. Urinary
non-nicked hCG is purified from the CR127 preparation of
pooled normal pregnancy hCG. Conversely C5 is recognized with
lower affinity by the B109-B108* assay, which has primary
15  specificity for the hCG isoforms of later pregnancy.

We have developed a method to directly profile changes of hCG
isoforms in serum or urine throughout pregnancy. Two IRMAs
for hCG are employed, each based on monoclonal antibodies to
20  different hCG epitopes. The B109-B108* assay is a commonly
used intact hCG assay to the heterodimeric-dependent epitope.
A new assay, B152-B207*, is most likely sensitive to the
carbohydrate portion of hCG carboxyterminal peptide. The same
standard non-nicked hCG was used in both assays. Non-nicked
25  hCG was employed since the B109 assay reacts poorly with
nicked forms of hCG while the B152 assay does not
discriminate between nicked and non-nicked forms of the
hormone. The B152 assay detected with greatly enhanced
sensitivity hCG isoforms which appear earlier in pregnancy
30  than isoforms measured by the B109 assay (O'Connor et al.
1998). Prior to development of the new immunometric assay
system described in this report, it was not possible to
readily discern the changes in hCG isoforms from very early
pregnancy to mid pregnancy. The only available procedure for
35  examining these changes was isoelectric focusing of every
patient specimen followed by immunoassay of every focused

fraction (Berger et al. 1993; Ulloa-Aguirre et al. 1990). The IEF pattern reflects the heterogeneity of the charged sugar, sialic acid which varies with the multi-antennary structures of the carbohydrate moieties in which sialic acid is the

5  terminal sugar. Although we do not yet know the precise nature of the isoform epitopes being measured, the evidence for carbohydrate discrimination is based upon the hyperglycosylated structure of the immunogen, C5, used to develop the B152 monoclonal antibody and the antibody's

10  reactivity with the hCG isoforms found in the JAR choriocarcinoma cell line.   C5 hCG was isolated from a choriocarcinoma patient and has been thoroughly characterized as to its protein and carbohydrate content and structure (Elliott et al. 1997).   It has been shown that C5 (and hCG

15  from other choriocarcinoma subjects) differ in the protein moiety mainly by the presence of an increased number of nicked sites and by increased glycosylation relative to the hCG of normal pregnancy.   In comparison with the hCG of normal pregnancy, choriocarcinoma derived hCG has increased

20  fucosylation of the N-linked biantennary oligosaccharides in the beta subunit.   In addition, the O-linked oligosaccharides in preparation C5 (a form of hCG produced from a single patient with choriocarcinoma) has a 100% tetrasaccharide core on the COOH-terminal region of the beta subunit. Normal mid

25  pregnancy hCG has only 10-20% of this structure (Elliott et al. 1997). These observations, plus our own determination that the hCG synthesized by the JAR choriocarcinoma cell line provides a B152/B109 isoform ratio similar to that observed in early pregnancy, leads us to the conclusion that in very

30  early pregnancy, the developing trophoblast secretes an isoform of hCG which resembles that produced in choriocarcinoma.

We have also tested recognition of pituitary hCG since its

35  N-Asn carbohydrates differ somewhat from those of placental hCG, bearing a closer resemblance to those of hLH which have

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both sialic acid and sulfate groups (Birken et al. 1996). The
carbohydrate structure of the b COOH-terminal portion of
pituitary hCG is not yet known. Since B152 did not recognize
any substantial differences between pituitary and placental
5 hCG (Figure 3), differences in N-Asn recognition are
unlikely. In terms of the COOH-terminal carbohydrates, it
appears that pituitary and placental hCG (mid-pregnancy
isoforms) may be similar, assuming the O-linked carbohydrate
on the C5 antigen is part of the epitope of B152.
10

**Example 2:       B152/B109 Ratio Predicts Pregnancy Outcome**

The B152/B109 ratio measured in urine samples throughout the
pregnancy

15

The relative concentrations of hCG isoforms in 103 normal
pregnancy urine samples (5-39 weeks post last menstrual
period - LMP) were determined by two immunometric assays
(B152-B207* and B109-B108*). Both because of the wide range
20 of hCG concentrations in different samples, even at the same
gestational age, and because neither of the assays is totally
specific for the two (or more) families of hCG isoforms
present, we find that presenting the data as a ratio of the
observed two isoform groups more clearly delineates the
25 change in isoform content as pregnancy progresses. This
calculated ratio is shown in Figure 4. In weeks 5-8 of
pregnancy, the ratio of B152/B109 isoforms ranged between 6.2
and 1.3, indicating a predominance of the B152 isoform(s) in
early pregnancy. During the 10 to 12 week period, the ratio
30 ranged from 1 - 0.2, indicating that an inversion in hCG
isoform content is occurring as pregnancy progresses. This
decline in the ratio continues, ranging from 0.54 - 0.08 in
the 15 - 18 week period and reaching an inflection point at
29 weeks. At that time, the ratio reached a value of around
35 0.06 after which the ratio displayed a rise to a range of 0.2
- 0.07 in the 37 - 39.5 weeks of gestation time period.

Statistical analysis involved fitting the log transformed ratio data to second and third order polynomial regression models. Since the third order term was not significant (likelihood ratio $c^2(1)=1.32$, $P=0.25$), the second order model

5 was used ($r^2=0.793$). The log B152/B109 ratio reached an inflection point at LMP=29 weeks, based on this model.

The B152-B207* values reflect a measurement of the B152 isoform in terms of later pregnancy hCG equivalents, not in

10 absolute quantities. It must be emphasized that the "absolute" concentrations measure in the B152 assay cannot be compared with the results of the B109 assay on an equimolar basis since the potency of the hyperglycosylated isoform is much higher in the B152 assay vis-à-vis the

15 standard, i.e. normal later first trimester pregnancy hCG. The actual molar values of this isoform are on the order of tenfold less than those recorded in the assay. For this reason we have chosen not to analyze absolute molar quantities of the two analytes but only the ratio of the two

20 measurements.

Even in normal pregnancy, the hCG values obtained vary widely according to the characteristics of the immunological reagents employed (Cole and Kardana, 1992; Cole et al. 1993).

25 We hypothesize that the two assays described in this report primarily detect hCG isoforms at opposing ends of this spectrum, each primarily recognizing a subset of closely related molecules in the continuum of early to later pregnancy hCG molecular forms.

30

We have retained the use of normal pregnancy hCG as the standard in B152-B207* assay, despite its decreased affinity in this antibody configuration. The reasons for this include the limited and unrenewable supply of C5 (which was isolated

35 from the urine of a single patient) and the variability in

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data which would result from investigations using different standards. The consequences of this choice are that the early pregnancy hCG isoforms have markedly increased immunopotency over that of normal pregnancy and hence their

5 molar quantities are overestimated in this assay. We use this difference in affinity to our advantage by employing a ratio of the molar results of two assays (B152 and B109). Either assay taken alone obscures this change due to the wide excursion of hCG values which occur in normal pregnancy.

10

Others have documented progressive changes in hCG isoforms throughout pregnancy. Skarulis et al. found that the fucose content of both intact hCG and also its free beta subunit increased as pregnancy progressed (Skarulis et al. 1992).

15 Diaz-Cueto et al. investigating the isoelectric focusing pattern of circulating hCG throughout pregnancy, found that in early pregnancy, more than 80% of the hCG isoforms were acidic. This fraction decreased to less than half (47%) late in the third trimester (Diaz-Cueto et al. 1996). In

20 contrast, Wide and Hobson found that the hCG of early pregnancy was more "choriocarcinoma-like" by virtue of its greater biological activity than the hCG of normal pregnancy (Wide and Hobson, 1987). Fein et al., in a study which employed gel filtration determined that first trimester hCG

25 was a larger size than that of the third trimester. Treatment with exoglycosidases eliminated the size differential, indicating that the first trimester hCG was more highly glycosylated (Fein et al. 1980).


30 <u>The B152/B109 ratio in matched serum/urine samples in the first and third trimesters of pregnancy compared with hCG from JAR cells.</u>


The B152/B109 ratio in serum is analogous to that found in

35 matched urine samples and undergoes a similar change as pregnancy progresses (Figure 5). The B152/B109 ratio in the

cell supernatant from JAR cells (a choriocarcinoma derived cell line) was similar to that of early pregnancy.

The B152/B109 ratios of both serum and urine hCG
5  concentrations are significantly higher in the first trimester as compared to the third trimester of normal pregnancies (Table 2). Significant differences between serum and urine hCG concentration ratios as well as log transformed ratios in early (5-6 weeks) and late (36-39 weeks) gestation
10  were evaluated by paired t-tests (Table 3). In both the first and third trimesters, urinary B152/B109 ratios were significantly higher than serum ratios, indicating that there was a preferential clearance of the B152-recognized isoform into urine, regardless of the relative concentrations of the
15  two isoforms.

**TABLE 2.**

Analysis of the B152/B109 ratio in serum and in urine in the first vs third trimesters of pregnancy.

| Measure | T-test(df) | $P$ |
|---|---|---|
| Serum, ratio B152/B109 | t(11)=6.65 | 0.0001 |
| Serum, log(ratioB152/B109) | t(23)=21.61 | 0.0000 |
| Urine, ratio B152/B109 | t(11)=4.64 | 0.0007 |
| Urine, log(ratioB152/B109) | t(15.7)=16.85 | 0.0001 |

40

**TABLE 3.**

Analysis of the B152/B109 ratio in serum vs urine in the first and third trimesters of pregnancy.

| Gestational age | Measure | Paired-t (df) | P |
|---|---|---|---|
| 5-6 weeks | Ratio B152/B109 Log(ratioB152/ B109) | t(11)=3.25 t(11)=6.25 | 0.0077 0.0001 |
| 36-39 weeks | Ratio B152/B109 Log(ratioB152/ B109) | t(10)=5.47 t(10)=7.14 | 0.0003 0.0001 |

In urine samples from IVF patients (1-4 weeks post embryo transfer - ET) the B152/B109 ratio was again between 2-8 and decreased as pregnancy progressed (Figure 6), similar to that observed in natural conceptions. The effect of pregnancy

5 duration with respect to outcome variables could best be represented by a linear or quadratic function. ANCOVA models including the second order week were fitted to the general equation: Outcome= (effect of time post ET)+(effect of diagnosis). After an appropriate ANCOVA model was determined,

10 the least square means (adjusted for week post ET effect) were compared among the normal pregnancy, ectopic pregnancy and spontaneous abortion populations (Table 4). The log transformed values of both B109-B108* and B152-B207* measured hCG forms discriminated both ectopic pregnancy and

15 spontaneous abortions from normal pregnancy (P=0.0001). The ratio of the log transformed values discriminated abortion from normal pregnancy (P=0.016). However, neither the ratio of B152/B109 nor the log of that ratio discriminated either of the pregnancy disorders from normal pregnancy.

20

A significant number of spontaneous abortions and ectopic pregnancies occur in IVF pregnancies. We did not find a difference in the ratio of the isoforms between either of

41

these two categories as compared to normal controls, possibly
a consequence of low statistical power. However a significant
difference was found between the B152 hCG isoforms levels in
normal pregnancy and spontaneous abortion. This supports our
5  previous finding in early pregnancy loss, where diminished
or absent levels of the B152 isoforms characterized an early
pregnancy loss (O'Connor et al. 1998).

**TABLE 4.**
IVF patients: analysis of covariance of hCG isoforms among
normal pregnancy (np), ectopic pregnancy and spontaneous
abortion as a function of gestational age.

| Outcome | [c]Adjusted $R^2$ | [e]-F | P | [d]-Pairwise Difference |
|---|---|---|---|---|
| [a]Log (ratio B152/B109) | 0.51 | 0.89 | 0.41 | none |
| [a]Log (B109-B108[+]) | 0.56 | 21.33 | 0.0001 | np vs abortion & ectopic |
| [b]Log (B152)/log (B109) | 0.45 | 4.34 | 0.016 | np vs abortion |
| [b]Log (B152-B207[+]) | 0.50 | 26.94 | 0.0001 | np vs abortion & ectopic |

[a]-   ANCOVA model with 2nd order polynomial coefficient
       (or  parameter).

[b]-   ANCOVA   model   with   only   1st   order   (linear)
       coefficient.

[c]-   Adjusted $R^2$ is a $R^2$ adjusting number of coefficients
       on  the ANCOVA model so that comparisons of two $R^2$
       with  different ANOVA models with different number of
       coefficients are meaningful.

[d]-   "Pairwise difference" is based on t-test comparing
       the least-square means of outcome variables (after
       adjusting effect of week ET).

[e]-   Degree of freedom (df1, df2) for F-test are (2,82)
       for a model with only linear coefficient and (2,81)
       for  a  model  with  both  linear  and  2nd  order
       coefficient.

42

HCG analysis of trophoblastic disease samples

Trophoblast disease serum (17 samples) and urine (28 samples) were obtained from patients post therapy and hence contained
5 low hCG levels. Due to limited amounts of sample all of these specimens were run at a 1:10 initial dilutions. HCG levels in serum were low. The highest hCG concentration in serum was 202 fmol/ml in the B152-B207* assay, with a corresponding value of 148 fmol/ml in the B109-B108* determination. Six of
10 seventeen samples in serum had detectable levels, with 4/6 having a higher value in the B152-B207* assay. Of the 15/28 positive urine samples however, 14/15 had higher levels in the B152-B207* assay than in the B109-B108* assay, with the highest hCG value being 20000 fmol/ml in the B152-B207* assay
15 and 18715 fmol/ml in the corresponding B109-B108* assay. Due to the small sample size, no statistical treatment was performed on this data, but even in these post-treatment patients the B152/B109 ratio was $\geq 1$, which corresponds to the early pregnancy hCG isoform ratio.
20

The specimen limitations discussed above precludes our reaching any definitive conclusion on the analysis of trophoblastic disease samples. However it appears as might be anticipated that the B152 assay is more sensitive than
25 B109 assay in detecting hCG immunoreactivity in the blood and in the urine of trophoblastic disease patients, even after treatment.


Chromatography of First Week of Gestation Pregnancy Pool.
30 In order to determine whether the B152-B207* assay recognized other forms of hCG associated immunoreactivity in addition to the intact hCG molecule, specimens were pooled. FPLC on tandem Superose 12 columns followed by immunoassay of the fractions for all of the characterized forms of hCG revealed
35 that only the intact hCG molecule (or hCG free beta subunit) gave a signal in this assay (See Figure 8). There were no

lower molecular weight fragments identified by the B152-B207*
assay. The hCG free beta analyte was measured in the 159
urines described in Figure 6 and was found to make a
negligible contribution to over all hCG immunoreactivity in
5 these specimens.

Molecules recognized by monoclonal antibody B152 in urine and
pituitary extracts. In order to define the nature of the hCG
isoforms recognized by B152, high resolution gel filtration
10 columns of both pituitary extracts and postmenopausal urine
concentrates were used (See Figure 8). The rationale for use
of pituitary extracts is to determine cross-reactive
molecules, specifically those which are glycosylated, which
are plentiful in pituitary which contains the entire family
15 of glycoprotein hormones, hLH, hTSH, and hFSH as well as free
subunits and the pituitary form of hCG. Two peaks are
detected in both of these cases. Only one peak was detected
in similar studies of pregnancy urine concentrates as
described earlier. In the pituitary, it is likely that the
20 larger molecule is pituitary hCG (70K) while the smaller
sized molecule is hLH. Since hLH is present at 100x or so
as compared to pituitary hCG, the apparent similar
concentration of immunoreactivity indicates that B152 has
reduced cross-reactivity to hLH as compared to hCG.
25 Likewise, both hCG and hLH occur in postmenopausal urine,
again with much more hLH than hCG and the B152 pattern is
similar to that of the pituitary extract. These results show
that B152 is generally hCG specific except for cross
reactivity to hLH (as shown by standard cross-reaction
30 studies in Table I) and that its carbohydrate specificity is
both to the protein portion as well as to the carbohydrate
moieties of hCG (and to a lesser extent of hLH) since it does
not react with the multitude of other glycoyslated proteins
present in the pituitary nor with those in postmenopausal
35 urine except for hCG or hLH-related molecules.

44

Serum and urine specimens were analyzed using two assays,
B109-B108*, and B152-B207+, which recognize the difference in
molecular isoforms of hCG.  See Table I.  The *in vitro*
bioassay for hLH/hCG is described above.  (See Figure 1).
5 Results are indicated in Figures 6-11.  The immunometric
assay employs 96-well microtiter plate technology.  The
coating antibody, at a concentration determined to provide
the most satisfactory combination of sensitivity and range,
is applied to the microtiter wells (Immulon IV, Dynatech
10 Laboratories) in carbonate buffer (0.2M, pH 9.5).  The plates
are incubated with the coating solution at 4°C, overnight,
then aspirated, washed with washing solution (0.05% Tween,
0.15N NaCl), and blocked with a 1% solution of BSA (three
hours at room temperature).  The BSA solution is aspirated
15 and the appropriate hCG standards (200μL/well), in buffer B
(PBS/0.1% bovine IgG/0.1% sodium azide), or in  hCG free
serum (Chemicon, Inc.), or hCG free urine, as appropriate to
the specimen matrix, and specimens are added to the wells.
The plates are sealed with plate sealers, and incubated
20 overnight at 4°C. The controls, specimens, and standards are
then aspirated, the plates washed 5 times with washing
solution, and iodinated detection antibody in buffer B (200
uL/well, 100,000 cpm/well) added and incubated overnight at
4°C. The wells are again aspirated, washed 5 times with
25 washing solution, separated and counted (Packard Cobra gamma
counted). Values are interpolated from a smoothed spline
transformation of the count data. This assay procedure, as
well as assay validation has been previously reported
(O'Connor, J. F., et al., 1988).

30

Creatinine analysis, when urine values are normalized to
creatinine, is performed in a microtiter plate format
following a modification of the Taussky procedure (Taussky,
H. H., 1954).

35

Descriptive statistical and graphical methods are used to measures of serum and urine samples from normal healthy pregnancies to identify the distributions a) between patient first trimester average B152 levels, B109 levels and
5 B152/B109 ratio; b) between patient variability in time to B152/B109 ratio reaching 1.00; and c) between patient variability in time to B152/B109 ratio declining by 1/3rd from first trimester maximum levels. The variability in the timing of the crossover in the ratio of these two analytes
10 provides an empirical basis from which to estimate the value of these markers as biochemical signatures of a viable third trimester fetus.

Comparison of the assay profile of healthy normal pregnancies
15 to those of unsuccessful pregnancies from failed IVF implantations, two non-parametric hypotheses are available: 1) the proportion of pregnancies in which the B152/B109 ratio falls below 1.00 is no different in healthy normal and unsuccessful IVF pregnancies; 2) the proportion of
20 pregnancies in which the B152/B109 ratio declines by 1/3rd from first trimester maximum levels is no different in healthy normal and unsuccessful IVF pregnancies. These hypotheses can be tested as a difference between two proportions. For example, a comparison of week 14 vs. week
25 9, week 13 vs. week 6, week 12 vs. week 5 or week 11 vs. week 4 pregnancies to show a reversal of the B152/B109 ratio in healthy normal pregnancies and unsuccessful IVF implantations, respectively. The power analyses apply to an outcome defined as the time at which the B152/B109 ratio
30 declines by 1/3rd from first trimester maximum levels, although this outcome would necessarily provide earlier detection of pregnancy failure than the reversal of the B152/B109 ratio. Patterns of results less discriminantly different from these indicate a rejection of the dichotomous
35 outcome of B152/B109 ratio reversal as a clinically meaningful marker of pregnancy failure.

Alternatively, the same two non-parametric hypotheses can be recast as parametric hypotheses by considering the timing of the biochemical events within the assay profile of healthy normal pregnancies and unsuccessful pregnancies from failed

5 IVF implantations: 1) the time at which the B152/B109 ratio falls below 1.00 is no different in healthy normal and unsuccessful IVF pregnancies; 2) the time at which the B152/B109 ratio declines by 1/3rd from first trimester maximum levels is no different in healthy normal and

10 unsuccessful IVF pregnancies. Of course, the objective is to provide an empirical basis from which clinicians may counsel their patients. Thus, it is important to adopt a logistic model for this component of the data analysis. With pregnancy success as the outcome, logistic models allow the

15 estimation of the (symmetrical) hypothesis of increase in risk of pregnancy failure for each additional week where either the B152/B109 ratio has failed to decline by one third from first trimester baseline maximum values or the B152/B109 ratio has failed to become less than 1.00 (measured in

20 weeks). The logistic model enables specification of the time at which results indicate a particular pregnancy exceeds an a priori defined likelihood of failure, given assay data regularly available during pregnancy, and allows incorporation of other risks for pregnancy failure in the

25 same data analytic framework to assess the relative contribution of threats to pregnancy loss. The Cox proportional hazard model may be used to examine predictors of the crossover rates. Mixed effects models can also analyze repeated measures of the B152/B109 ratios taken during entire

30 cycles. These models are particularly useful since they allow inclusion of incomplete and imbalance data (i.e. data with missing values and unequal timing of data collection), to estimate effects of time-varying covariates, to model dependency structure of repeated measures and to model

35 possible heterogeneity of the ratio measures within each experimental group.

47

<u>B152 hCG isoforms isolated from early pregnancy urine and
determination of their protein and carbohydrate structures</u>.
Using the already developed scheme of concentration and
immunoaffinity extraction of urine, hCG molecules are
5  isolated from urine collected from women in early pregnancy
for both protein and carbohydrate analyses.  According to one
approach, molecules are isolated from HPLC fractions,
digested with proteases before and after reduction of
disulfide bonds, examination of the resultant peptides by
10 mass spectrometry and/or sequence analysis, isolation of
carbohydrate moieties after glycosidase digestions and
determination of carbohydrate structures by a combination of
specific glycosidases and retention times on specialized
anion exchange columns as compared to know branch-chain
15 oligosaccharide standards.  In a similar approach, the final
purification stage for the isolated hCG isoforms is SDS gel
electrophoresis.   Both protease digests and glycosidase
digests are performed on the blotted and cutout band.  This
method results in greater purity of the protein and less
20 artifactual errors due to contamination by carbohydrates
which are not in the purified protein but are derived from
outside contaminants.


<u>Carbohydrate compositional analyses and oligosaccharide</u>
25 <u>branched chain identifications</u>.   The MALDI TOF mass
spectrometric method may be used to confirm oligosaccharide
structures   by   using   specific   glycosidases   on   the
glycopeptides and determining the change in molecular weight
as the sugars are digested off the glycopeptide.  Only the
30 hCG beta COOH peptide can be expected to contain O-linked
sugar moieties.  These are of special interest since it is
thought that B152 has significant reaction with this region.
The structures of this region can be determined in a similar
fashion using enzymes that specifically release O-linked
35 glycans.   The O-linked structures has been previously
examined using standard reference pregnancy hCG (Cole, L. A.,

et al., 1985). The O-linked branched chain structure are determined by a similar strategy using the Dionex chromatographic system as well as specific glycosidases on the C-terminal glycopeptides and Mass Spectrometry. In one
5 study (Elliott, M. M., et al., 1997), these techniques were used to elucidate the carbohydrate structures of CR series hCG preparations (standard urinary pregnancy hCG) and compared them to the structures of patient samples such as C5 which was the immunogen employed to generate antibody
10 B152. It was found that C5 contained significantly more mono and tri-antennary (2X mono and 3X tri-structures than the CR preparations) on the N-Asn residues. It was also found that more tetrasaccharide structures were on the hCG COOH-terminal peptide O-Serine residues in the choriocarcinoma hCG isoform
15 than in the CR preparations.


Biological activity and metabolic clearance of hCG isoforms.
Biological activity is a function both of molecular structure and half-life in the circulation, which can be influenced by
20 structure. Alterations in carbohydrate/sialic acid content of the glycoprotein hormones are thought to be responsible for the changes in hCG biological/immunological activity observed throughout pregnancy. In addition, signal transduction at the receptor is influenced by the pI of the
25 hCG isoform and the presence or absence of carbohydrate. Thus, it is valuable to examine both receptor binding and biological activity in vitro and, in order to determine the mechanism of action, to distinguish receptor binding and signal transduction as well as relative potency of signal
30 transduction along with in vivo bioactivity determinants such as circulating half life. Studies, including clearance rates, are performed on B152 hCG isoforms of early successful pregnancy, hCG from third trimester pregnancy, and the reference urinary hCG preparation, CR 127.
35

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**Example 3**: **B152 and B151 immunoreactivity in non-trophoblastic malignancy.**

With the exception of trophoblastic disease and testicular cancer, hCG is expressed in the blood of about 20% of
5  patients with all other types of cancer(Hussa, R. O., 1987). HCG beta core fragment in the urine has a significantly higher level of expression, especially in gynecological malignancy. Since the B152 antibody was developed to a form of hCG produced in a malignancy, it was of interest to
10 examine the expression of B152 and nicked hCG immunoreactivity (B151) in non-trophoblastic malignancy. Accordingly, blood and urine derived from men undergoing chemotherapy for prostate cancer or women for ovarian cancer were evaluated for the expression of hCG isoforms in plasma
15 and urine. It is significant that in prostate cancer, B152 hCG immunoreactivity is found in the blood and urine of prostate cancer patients in instances when there is no hCG detected by B109-B108*. In ovarian cancer patients evaluated, there is evidence of nicked hCG in the blood, even
20 in the absence of both B109 and B152 immunoreactivity. Neither of the above groups demonstrated the presence of hCG immunoreactivity when the standard pregnancy derived hCG assay was employed. It is reassuring to find that nicked hCG, the existence of which has been documented by several
25 investigators, can be found and reliably measured in a clinical setting.

**Experimental Discussion**

In the course of these studies, a potentially important new
30 signal was observed in the urine of women early in pregnancy, namely an epitope of a form of hCG which may indicate the likely success of carrying a pregnancy. Likewise, absence of this signal may indicate that EPL will occur. Since EPL can be a very sensitive marker of environmental toxins
35 (Hakim, R. B., et al., 1995) and is frequently used as an

epidemiological marker of exposure, the finding of this epitope provides a powerful tool for monitoring the safety of the environment. In addition, this assay facilitates increasing the success rate of IVF infertility programs since
5  the predictive value of the new measuring system would rapidly indicate successful approaches. Described herein is the novel and completely unexpected finding that successful pregnancies display a high content of unique isoforms of hCG that are maintained for the first few weeks of pregnancy and
10 then rapidly decline as pregnancy progresses. Based on properties of the immunoassay system, it is hypothesized that these hCG isoforms may be hyperglycosylated. This is a striking observation never reported nor suspected earlier. Carbohydrate analyses (Elliot, M., 1997) demonstrate that C5
15 hCG employed as immunogen for antibody B152, contains two times the monoantennary content and three times the tri- antennary content of branch chain sugars as compared to the CR series of natural pregnancy urinary hCG. In addition, the O-linked carbohydrates are mostly tetrasaccharide instead of
20 disaccharide in C5 as compared to CR 127 hCG. (CR 127 hCG is similar to the WHO preparation, the third international hCG standard, which was CR 119 hCG, prepared by Canfield and Birken twenty years ago but still in use today)(Birken, S., et al., 1991a). B152 recognizes C5 hCG much better than
25 nicked CR127 hCG or non-nicked CR 127 hCG (Birken, S., et al., 1993). In addition, JAR cell type hCG is known to contain a similar array of carbohydrate moieties. It was found to be recognized by B152 similar to the early pregnancy isoforms in healthy pregnancies. The observation that the
30 hCG isoform produced by JAR cells in culture (B152/B109 ratio) is similar to that found in early pregnancy hCG isoforms supports the hypothesis that the production of a type of hCG with a particular glycosylation pattern is a prerequisite for a viable pregnancy. This glycosylation
35 pattern is not characteristic of the hCG of later pregnancy.

51

A variety of pregnancy disorders are testable. One category
of patients consists of those women who experience a high
rate of recurrent abortions. Even in populations with no
known fertility problems, the total rate of pregnancy loss
5 is 32% (EPL plus clinically recognized abortion) (Wilcox, A.
J., et al., 1988). The risk of recurrent abortion increases
with the number of spontaneous abortions experienced in the
past, reaching an incidence of 32% after three consecutive
abortions. (Hill, J. A., and Anderson, D. J., 1990). Probable
10 causes of recurrent spontaneous abortion, comprising genetic,
infectious, hormonal imbalance, or immunologic factors can
be established in less than 60% of all spontaneous abortions,
leaving 40+% of spontaneous abortions with a completely
unestablished etiology. These facts, taken together with
15 reports establishing that the administration of exogenous hCG
can be an effective therapy in subjects with a history of
recurrent spontaneous abortion (Quenby, S., and Farquharson,
R. G., 1994; Harrison, R. F., 1985) lends support to the
hypothesis that a disproportionate production of the
20 ineffective isoforms of hCG in early pregnancy is a causal
factor in both early pre-clinical loss as well as in
spontaneous abortion.

A second category includes women undergoing embryo transfer.
25 These patients provide several distinct advantages: The
patients undergoing this procedure are not treated with crude
hCG preparations, making measurement of hCG isoforms easy and
decisive since all hCG forms derive from the embryo none from
any injected hCG preparations. Second, is the opportunity
30 to monitor the nature of the isoforms from day 9 of a
successful pregnancy. Third, is the ability to obtain large
volumes of urine to purify the early pregnancy isoforms to
determine their structures. Fourth, since pregnancy loss is
from 50% to 70% in this population, the loss can be defined
35 as due to lack of the essential hCG isoform recognized by
B152 or due to other causes. Comparison of early pregnancies

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in populations of women not undergoing *in vitro* fertilization
procedures with those undergoing embryo implantation can,
thus, assess whether pregnancy loss situations present
similar or different patterns of hCG isoforms during the
5 process.  The mechanism of pregnancy loss in the general
population as compared with the much higher rate of embryo
loss in IVF programs may be different. Additionally, it has
been established that the hCG produced in choriocarcinoma has
differences in carbohydrate structures, sialic acid content
10 and biological activity (Wide, L., and Hobson, B., 1987;
Elliot, M., et al., 1997; Hussa, R. A., 1987). Since both the
B151-B604* and B152-B207* assays incorporate monoclonal
antibodies raised against an immunogen derived from
choriocarcinoma, specimens may be evaluated from patients
15 with gestational trophoblastic disease in order to determine
whether the above assays recognize the hCG produced in these
conditions with greater sensitivity and specificity than do
assays based on the hCG of normal pregnancy, as is apparently
the case for the hCG produced in testicular and ovarian
20 cancer.


There are few reports of changes of carbohydrate content of
hCG-related molecules during pregnancy.    Blithe and
colleagues studied free alpha subunit of hCG whose
25 carbohydrate content differs from that of alpha within hCG
by additional carbohydrate antennae and fucose.    The
carbohydrate of free alpha becomes increasingly complex in
terms of more branches and higher content as pregnancy
proceeds.   It has also been reported that the quantity of
30 fucose increased in both hCG and in free alpha as pregnancy
proceeded (Skarulis, M. C., et al., 1992).   Thus, the
literature indicates increasing content and complexity of
carbohydrate of hCG and free alpha subunits.   However,
immunological data using the B152 monoclonal antibody,
35 implies a progression to simpler carbohydrate content during

53

pregnancy.    Since   the   beta   COOH-region's   O-linked
carbohydrates may be involved in the epitope recognized by
B152, it is conceivable that the carbohydrate structures of
this region may be altered in a different pattern from the
5 N-linked glycans studied by Blithe and colleagues (Skarulis,
M. C., et al., 1992; Blithe, D. L., and Iles, R. K., 1995).
Data from Skarulis et al. indicate that heterodimeric hCG may
contain additional fucose but do not provide data that this
late pregnancy hCG becomes hyperglycosylated as does free
10 alpha.

Other studies indicated that the forms of hCG during EPL
likely differ in biological activity from those hCG isoforms
in successful pregnancies (Ho, H.-H., et al., 1997).  The *in
15 vitro* bioassay employed in those studies are unsuitable for
large-scale   studies   and   are   not   as   reliable   as   the
immunoassays described herein.   Furthermore, it is likely
that *in vivo* assays may give different results since *in vitro*
and *in vivo* assays sometimes give completely disparate
20 results.  In this case, *in vivo* and clearance assays are most
important in order to identify whether the hCG isoforms are
truly more potent in the whole animal and to identify the
reasons for the increased potency.  Thus *in vitro* and *in vivo*
bioactivities of the early pregnancy isoforms of hCG are
25 highly significant.

Carbohydrate differences is a widely accepted explanation for
variations in biological to immunological ratio such as the
forms observed by various studies of EPL (Ho, H.-H., et al.,
30 1997).  Various studies (Grotjan, H. R. J., and Cole, L. A.,
1989;   Hoermann,   R.,   1997;   Stanton,   P.   G.,   et   al.,   1993;
Szkudlinski, M. W., et al., 1995, Thotakura, N. R., et al.,
1994;   Szkudlinski,   M.   W.,   et   al.,   1993),   have   shown   that
sialic   acid   differences   are   an   explanation   for   such
35 heterogeneity   in   biological   activities   of   glycoprotein

54

hormones.  These studies have also confirmed the dogma that
*in vitro* biological activities can yield the opposite results
from *in vivo* studies because of altered metabolic clearance
rates in the latter studies.  Thus, more acidic (more highly
5 sialylated) forms of gonadotropins are more biopotent in the
whole animal because of prolonged circulating half-lives.
The same molecules may appear less potent in *in vitro* assays
due to greater acidity, greater negative sialic acid content.
Hoermann et al. (Hoermann, R., et al., 1997) demonstrated the
10 exclusion of many of the acidic circulating hormone forms
from the urine, thus, prolonging their half-lives.  The pI
pattern of normal pregnancy as well as trophoblastic cancer
hCG in serum is quite different from that of urine.  Since
the studies described herein indicate that EPL hCG isoforms
15 have reduced *in vitro* biological activity, this finding
cannot be explained solely by what is known of biological
activity and sialic acid content.  Early pregnancy isoforms
recognized by monoclonal antibody B152 may be more potent *in
vivo* by virtue of prolonged half-life they may then display
20 increased signal transduction at the receptor as well.  This
may be explained by a hyperglycosylated form of hCG which is
not hypersiaylated.  In this case, the extra sugar portion
would help prolong circulating half-life of a more basic pI
form of hCG which also has increased *in vitro* bioactivity.
25

**Example 4:**  **Diagnosis of Gestational Trophobloast Disease**.
An important application of the B152 (early hCG isoform)/B109
(late hCG isoform) ratio analysis described herein above is
in the very early (and facile) diagnosis of gestational
30 trophoblast disease.  Examples of gestational trophopblast
disease include choriocarcinoma or hydatidiform mole.  In
normal pregnancy, the ratio of B152/B109 of the two isoforms
of hCG rapidly decreases, eventually inverting.  In
gestational trophoblast disease including choriocarcinoma or
35 hydatidiform mole, the ratio is initially higher than found

55

in normal pregnancy, but does not diminish during the course
of the apparent pregnancy. This approach provides a highly
sensitive and specific diagnostic marker for gestational
trophoblast disease.

5

Other pregnancy disorders in which hCG levels are abnormally
high or abnormally low include Down's syndrome or other
aneuploid pregnancies, ectopic pregnancy, preeclampsia, and
intra-uterine growth retardation. Because the hCG production
10 in these conditions is quantitatively abnormal compared with
normal pregnancy, an altered ratio of the hCG isoforms
identified by B152 (early hCG isoform) and B109 (late hCG
isoform) can be detected. Thus, the dual isoform analysis
(B152/B109) further provides a method for diagnosing
15 pregnancy disorders and gestational trophoblast disease.


**Materials and Methods**

Hormones

The non-nicked hCG isolated from the CR127 preparation of hCG
20 was used as a standard in both assays (Birken et al. 1993).
The pituitary hCG was isolated as described (Birken et al.
1996). C5, a 100% nicked hCG having extra sugars on both N-
and O-linked carbohydrate moieties, purified from the urine
of a choriocarcinoma patient (Elliott et al. 1997), was
25 supplied by Dr. Laurence Cole (Yale University School of
Medicine). Although the C5 immunogen used in the development
of B152 antibody was 100% nicked hCG isoform (i.e. had
cleavages in the peptide backbone of loop 2 of the b subunit)
the antibody did not discriminate nicked from non-nicked hCG
30 (O'Connor et al. 1998).


The same serial dilutions of non-nicked hCG, pituitary hCG
and C5 were used for binding characterization in hCG assays.
Hormone concentrations of initial stock standards solutions
35 were determined by amino acid analysis.

56

## Immunoradiometric assays (IRMA)

The methodology used in the construction and validation of
the B109-B108* assay has been fully described elsewhere
5 (O'Connor et al. 1988). The B152-B207* assay has also been
characterized (O'Connor et al. 1998). Both assays were
performed with a slight modification of the published
procedure: the capture antibody was adsorbed onto the wells
of microtiter plates (Immulon IV, Dynatech, Chantilly, VA)
10 by incubating a 5 µg/ml solution (B109-B108* assay) or 25
mg/ml solution (B152-B207* assay) in coating buffer (0.2 M
bicarbonate, pH 9.5) overnight at 4 C. The coating antibody
solution was aspirated, the plates washed (wash solution:
0.9% NaCl, 0.05% Tween 20) and blocked with a 1% solution of
15 BSA in PBS with 0.1% sodium azide. Following incubation with
the BSA solution (minimum 3 hours at room temperature) the
blocking solution was removed, the wells again washed with
wash solution and 200 ml/well of the appropriate hCG
standards were added in phosphate buffer B (PBS with 0.1%
20 bovine gamma globulin and 0.1% sodium azide). After overnight
incubation at 4 C, the plates were again aspirated and
washed. The 200ml (50,000 cpm-100,000 cpm) of $^{125}$I- labeled
antibody was added to the wells which were again incubated
for 24 h at 4 C. The tracer was aspirated, the plates washed
25 with wash solution, the individual wells placed in glass
tubes and the radioactivity determined in a Packard Cobra
gamma counter. Doses were determined by interpolation from
a smoothed spline transformation of the data points.

30 All samples were stored frozen at -20 C prior to assay.
Because extreme values of sample pH may interfere with
antibody binding, the urine pH was adjusted with 1.0M Tris
(pH 9.0), 50µl/ml urine prior to assay, so that the final pH
was in the range of 7-7.4 (O'Connor et al. 1988). Intra-assay
35 variation was 6% for both assays, inter-assay variation was
12% for B109-B108* and 13% for B152-B207* assays. Sensitivity

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(least detectable dose, defined as +2SD from the zero
calibrator, was 1 fmol/ml for the B109-B108* assay and 2.2
fmol/ml for B152-B207* assay.

5 <u>Patients samples</u>

Urine samples from IVF patients were a gift from Dr. L. Cole.
They included spontaneous abortion (n=14, range of
gestational age 1.8-4.1 weeks from ET- embryo transfer),
10 ectopic pregnancies (n=7, gestational age 2.3-4 weeks) and
normal pregnancy controls (n=65, encompassing the range 0.6
to 5.4 week from ET). Some of the normal pregnancy urine
samples throughout the pregnancies were also obtained from
Dr. Cole. Others were obtained from the clinical practice of
15 collaborating physicians at Columbia Presbyterian Medical
Center (CPMC) (Total n=103). Matched serum/urine samples
from the first (n=12) and the third (n=11) trimesters were
provided by Dr. Amalia Kelly at CPMC. Trophoblast disease
serum (n=17) and urine (n=28) samples were obtained from Dr.
20 Cole, but were collected by Dr. Edward Newlands (Charing
Cross Hospital, London, UK). All specimen collection
protocols were approved by the appropriate Institutional
Review Board.

25 <u>Statistical Analysis</u>

Polynomial regression models of log transformed hormone
ratios were used to describe the relationship between the
change in ratio as a function of gestational age in normal
30 pregnancy. A paired t-test was used to evaluate the
relationship between matched serum and urine hormone ratios.
Analysis of covariance (ANCOVA) was used to describe the time
adjusted relationship of hormone values in ectopic pregnancy
and spontaneous abortion to those of normal pregnancy.

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## Urine Processing.

Twenty-four hour urine samples are collected from women undergoing embryo transfer as well as women in early natural pregnancy. The urine is refrigerated during the collection

5 procedure. After delivery of the urine to the laboratory, sodium azide is added to 1g/liter. Women undergoing *in vitro* embryo transfer are not pre-treated with hCG. Thus, all hCG which appears in their blood or urine is derived from the embryo (except for the small amounts of pituitary hCG present

10 in all people). Raw urine is freed from particles by centrifugation followed by Pellicon filtration through a 0.45 micron membrane. Next, the procedure is to concentrate the urine with a Pellicon (Millipore) system which concentrates as much as 30 liters to 500ml overnight (4°C) using a 3,000

15 MW cutoff membrane. Smaller volumes can be concentrated in less that two hours. Next, the urine is desalted and delipidated by passage through a large volume of Sephadex G25 in 0.1 M ammonium bicarbonate. This step greatly increases the binding of CG to immunoaffinity columns. The desalted

20 urinary concentrate is next size fractionated on the Pharmacia HiLoad Superdex 200 and the hCG and hCG subunit peaks are identified by specific immunoassays (O'Connor, J. F., et al., 1994) and the appropriate fractions are pooled and dried. The hCG and hCG subunits are purified from the

25 gel filtered urine concentrate by immunoaffinity on insolubilized hCG antibody columns as described but with the use of either 4M guanadine (0.1M tris acetate, pH 5) or ammonium thiocyanate as eluant to decrease loss of sialic acid from the hormone. Alternatively, hCG is purified by

30 conventional chromatographic procedures, anion exchange and hydrophobic chromatography. The subunits are separated on reverse phase HPLC using a 0.01M sodium phosphate, pH 5 buffer and acetonitrile, after incubation in 4M guanadine, 0.1M tris acetate, pH 5. A third method is final

35 purification and separation of the hCG subunits on SDS PAGE

electrophoresis followed by electroblotting to PVDF.  The
PVDF band can be subjected to protease digestion to release
peptides and glycopeptides which can be separated on reverse
phase HPLC in neutral pH 5 buffers.

5

Separation of Glycopeptides from Isolated hCG subunits.  To
facilitate isolation of the glycopeptides from the hCG
subunits, the subunits are both tryptic digested and the
products of digestion are separated on reverse phase HPLC
10 (using a pH 5 buffer).  This procedure results in removal of
the large beta COOH-terminal peptide which contains O-linked
sugars.  It also releases small, non glycopeptides from both
subunits (Pollak, S., et al., 1990, Birken, S., et al., 1987;
Birken, S., et al., 1986).  Next, the main disulfide-linked
15 core of each hCG subunit, is reduced and carboxymethylated,
and separated on reverse phase HPLC at pH 5.  At this stage,
large peptides are isolated, including the glycopeptides.
Each separated glycopeptide is redigested with trypsin and
re-separated on HPLC at pH 5.  These glycopeptides are next
20 employed for two different methods of sugar chain analysis.
One method is the approach of releasing the oligosaccharides
by enzymatic digestions uing PNGase F for the N-linked
glycans.  The released glycans can be separated from the
peptides by ethanol precipitation, desialyated with
25 neuraminadase, and separated directly on a Dionex Carbopac
PA-100 column.  Oligosaccharide standards are available from
Dionex, Oxford Glycosystems and other companies for
calibrating column elution times for various glycans (Hardy,
M. R., and Townsend, R. R., 1994, Rohrer, J. S., et al.,
30 1993, Weitzhandler, M., et al., 1993; Townsend, R. R., et
al., 1989).  Confirmation of the released structures is
obtained by performing carbohydrate compositional analysis
of eluted glycan peaks as well as performing digestions with
specific glycosidases and rechromatographing the modified
35 glycan on the Dionex system (Hardy, M. R., and Townsend, R.
R., 1994; Rohrer, J. S., et al 1993; Weitzhanlder, M., et

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al., 1993; Townsend, R. R., et al., 1989; Townsend, R. R.,
et al., 1991; Townsend, R. R., et al., 1989; Hardy, M. R.,
and Townsend, R. R., 1989; Townsend, R. R., et al., 1988;
Hardy, M. R., et al, 1997; Hardy, M. R., and Townsend, R. R.,
5  1988; Dionex, 1997; Spellman, M. W., 1990; Kumarasamy, R.,
1990). The newly modified glycan can be observed to elute at
the same time as the appropriate standard oligosaccaharide
and, in addition, the released monosaccharide can frequently
be identified as well (Dionex, 1997). Structure
10 determination is facilitated by the use of specific
glycosidases for branch chain cleavage as well as for
digestion of individual sugars from each of the branch
chains. For example, Endo H cleaves high mannose type and
hybrid oligosaccharide chains while glycosidase Endo F2
15 cleaves biantennary complex types and PNAase F cleaves tri
and tetra-antennary chains down to the N-Asn bond.


Competitive receptor binding and in vitro bioassay.
Bioassays are performed with recombinant-engineered CHO cells
20 transfected with the human receptor to LH/CG Cells are
maintained in Ham's F-12 medium, 4mM Glutamine, 400ug/ml G418
(Gibco), 5% fetal calf serum, 100IU/ml penicillin, 100ug/ml
streptomycin. The cells are removed from the flask surface
by versene only.
25

A competitive receptor assay constructed as follows: The
receptor binding assay mixture contains 100 ul of the
appropriate dilution of serum/urine samples or hCG dilutions
for standard curve, 100ul of $^{125}$-I-hCG (50,000-100,000cpm) in
30 buffer A(PBS/0.1%BSA) and 100ul of CHO cells (2x10$^5$ cells in
PBS). The mixture is incubated at 37°C with slight shaking
followed by centrifugation for 10 minutes at 750xg. The
supernatant is aspirated and the cell pellet is counted in
gamma-counter.
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*In vitro bioassay*. Transfected CHO cells are seeded (200,000 cells/well) into a 24 well plate in culture medium and incubated for 2-3 days until the cells reach confluence. Non-transfected CHO cells are included to monitor non-
5  specific response. The medium is removed and replaced with medium containing 1 mM isobutylmethylxanthine with appropriate dilutions of tested serum or urine. The plates are incubated at 37°C for two hours. The supernatant is removed, and the wells washed with Hank's balanced salt
10  solution. The intracellular cAMP is extracted with 95% ethanol, which is diluted 1:5, (or up to 1:40, depending on cAMP content) in assay buffer provided by the cAMP kit (New England Nuclear). cAMP assay is performed according to manufacturer's instructions. Response is normalized to well
15  protein content (BCA protein assay kit, Pierce, Rockford, IL).

*In vivo bioassay* is determined by the uterine weight assay in immature female mice, following the procedure of Wide and
20  Hobson (Wide, L., and Hobson, B., 1987). The mice are injected subcutaneously with one third of the total dose of gonadotropin on three consecutive days and killed 72 hours after the first injection. Uteri are dissected free from mesentery, fat and oviducts, blotted to remove intrauterine
25  fluid and weighed to the nearest 0.1mg. Five to ten mice are used at each of these dose levels. The hCG standard preparation used is a nicked hCG. This material may be run concurrently with specimens isolated from first and third trimester pregnancy. Sham saline injection may be used as
30  a control. The response signal is the log mouse uterine weight.

Clearance of hCG isoforms. The clearance of hCG is determined in the rat. Blood (200ul/sample) is obtained at
35  0, 120, 240, 360 and 480 minutes post injection, from an

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indwelling catheter in an catheterized external jugular vein, following the procedure described by Newman et al. (Newman, C. B., et al., 1985) and Brown and Hedge (Brown, M. R., and Hedge, G. A., 1972). Briefly, adult male Sprague-
5  Dawley rats (Charles River Laboratories, Wilmington MA), wt 175-225g, are given free access to food and water. Rats are handled for acclimatization for one week after arrival, and several days before the hCG infusion, the rats are cannulated under pentobarbital anesthesia. A 21 gauge stainless steel
10 cannula is inserted into the one external jugular vein. The placement of the catheter allows for the collection of blood from the unrestrained, unstressed rat. After the animals have recuperated from the cannula implacement , an hCG isoform is injected (10 µg/ml sterile saline) through the
15 cannulated vein. Blood samples are obtained at the four time intervals listed above. The blood is allowed to clot and the serum separated and stored at -80°C for immunometric assays specific for different hCG isoforms.

20 Clearance rate of the isoforms of hCG from the circulation of the rat are estimated by computer fitting the concentration data to an equation of the general form: Concentration=$Ae^{-\alpha t}$ +$Be^{-\beta t}$  at time t; A and $\alpha$ are parameters of the rapid component and B and $\beta$ are parameters of the slow
25 component. The metabolic clearance rate (MCR) is calculated as MCR= Dose/(A/$\alpha$+B/$\beta$) and the initial volume of distribution is calculated from $V_3$=Dose/(A+B). The MCR is normalized to body weight for statistical analysis, which is performed using ANOVA with Duncan's range test for determination of
30 significance (Cassals, J. W., et al., 1989).

Mice. The mouse species used in the experiments described herein are Balb/c mice, aged 12-20 weeks old and adult Sprague-Dawley rats of either sex. Mice used for the
35 production of monoclonal antibodies through ascites and for

the determination of *in vivo* biological activity as described. Balbc/c mice are used because hybridoma cell lines were developed using Balb/c splenocytes.

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## EXPERIMENTAL DETAILS FOR THE SECOND SERIES OF EXPERIMENTS

Human Chorionic Gonadotropin exists in blood and urine as a
variety of isoforms one of which contains peptide bond
cleavages within its beta subunit loop 2 is referred to as
5 nicked hCG (hCGn). This is hCG isoform appears to be more
prevalent in the urine of patients with certain malignancies
and possibly in some disorders of pregnancy. Until now, only
indirect immunoassays could be used to quantify hCGn. We
report the development of two monoclonal antibodies to a form
10 of hCGn isolated from a choriocarcinoma patient. This hCG
isoform was not only 100% nicked but also contained 100%
tetrasaccharide-core O-linked carbohydrate moieties in its
beta COOH-terminal region. Two-site immunometric assays have
been developed using these new antibodies, B151 and B152.
15 The former exhibits good specificity for hCGn independent of
the source of the hCGn, that form excreted by choriocarcinoma
patients or the form of hCGn form normal pregnancies. The
latter antibody, B152, is sensitive to the carbohydrate
moieties and possibly other differences in hCG isoforms but
20 not for nicking of the beta subunit. These two immunometric
assays provide potential novel diagnostic tools for direct
measurement of hCG isoforms which could not be accurately
quantified earlier before development of the assays using
these newly generated antibodies.
25

Human Chorionic Gonadotropin (hCG) is a glycoprotein hormone
produced by trophoblast cell of the placenta. The
measurement of this hormone in blood or urine is the basis
of all pregnancy tests. It is secreted by the trophoblast
30 starting very early in pregnancy and functions to maintain
steroid production by the corpus luteum until the placenta
takes over that function later in pregnancy (1). There has
been much recent interest in measurement of the hormone or
its subunits or fragments for purposes other than the
35 diagnosis of pregnancy such as monitoring of therapy for hCG-

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secreting malignancies (1), tests for indication of Down
syndrome or other genetically-abnormal pregnancies (2-11),
ectopic pregnancies (12), etc.   HCG appears in urine in a
variety of forms including free subunits, heterodimeric hCG
5 with peptide bond cleavages in loop 2 of its beta subunit
(known as nicked hCG), free nicked beta subunits, and the
beta core fragment (13-16). The nicked form of heterodimeric
in hCG has been reported to be present in blood as well as
urine and is known to have much reduced immunochemical
10 recognition by some antibodies directed to heterodimeric hCG
as well as greatly reduced biological activity (13, 16-17).
There are reported associations between increased nicking of
hCG with certain  hCG-secreting malignancies (18,19).


15 There are no satisfactory direct immunoassays for the nicked
form of hCG nor for the nicked form of free hCG beta subunit.
All measurements to date have been conducted with subtractive
assay procedures or immunoassays which include scavenger
antibodies (20-22).  We report here the development of two
20 antibodies of distinct specificity, using as immunogen the
form    of    hCG    produced    by    a    single    individual    with
choriocarcinoma.  This particular hCG isoform was 100% nicked
and hyperglycosylated both in its N and O-linked carbohydrate
moieties.  One resultant antibody, B151, displays significant
25 preference    towards    binding    to    its    choriocarcinoma    hCG
immunogen and does not recognize the nicked characteristic.
Immunoassays developed using B152 are of special interest in
their enhanced recognition of hCG isoforms more prevalent in
preeclampsia and Down syndrome (23,24).   B152 also appears
30 to detect an hCG isoform associated with healthy pregnancies
as juxtaposed to those pregnancies destined to fail early
which have little of this isoform (25).



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**Results:**

Characteristics of antibodies

A variety of hCG isoforms were employed to characterize the
new antibodies described in this report and the nomenclature
5 and characteristics of each of the reagents employed is
summarized in Figure 11. The carbohydrate groups in these
hCG isoforms as well as the percent nicking were analyzed in
an earlier study (26) and are directly relevant for defining
the nature of these new antibodies in this report.

10

Two antibodies designated B151 and B152 were selected by the
use of radiolabeled hCG isoforms, chorioCG C5 and pregnancy
hCG CR127. Each displayed preferential binding to C5 as
compared to CR 127 since this was the selection criterion.
15 However, upon performing liquid phase immunoassays and
calculating affinity constants, it was clear that these two
antibodies were very different in specificity (Figure 8).
It was found that antibody B151 had one order of magnitude
higher affinity both for C5, which is nicked and
20 hyperglycosylated choriocarcinoma hCG, and for CR127 hCGn
(813) as compared to CR 127 hCG or nick-free CR 127(814) (see
Figure 11 for reagent descriptions). B151 was clearly an
antibody with a strong preference for binding to various
forms of nicked hCG. Antibody B152 was different in that
25 although it displayed one order of magnitude preference for
C5 hCG over CR127 hCG, it recognized the nicked and non-
nicked forms of CR 127 hCG, hCG derived from normal
pregnancies, to an equal extent.


30 Liquid Phase Assays

Figure 8 shows potency comparisons of liquid phase
immunoassays of both B151 and B152 antibodies comparing
competitors: 1. standard CR 127 pregnancy hCG (which has a
20% content of nicked hCG); 2. C5 chorio CG (100% nicked and
35 hyperglycosylated); 3.813, nicked CG made from CR 127 by

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purification, and 4.814, non-nicked hCG derived from CR 127.
The labeled ligand was C5 chorio CG. It is apparent that
B151 (Fig 8A) shows a preference for nicked forms of hCG.
C5 chorio-CG or 813 hCGn bind with similar affinities. The
5 slightly lower potency of 813 hCGn may be ascribed to its 20%
contamination with non-nicked hCG. B152 only shows a
preference to C5, the hyperglycosylated choric CG (Fig 8B).
813 hCGn is no more potent a competitor than nick-free 814
hCG.

10

Immunometric Two Site Assays

A variety of two site antibody formats were tested. Figure
9 displays these results. It is apparent that B151 cannot
bind simultaneously with antibodies (designated by us a site
15 IV) (27) to the beta subunit and beta subunit core (B201 and
B204) nor with antibodies directed towards the determinant
which exists in heterodimeric hCG as represented by antibody
B109 (site III, to which A109 also belongs) (27). In
contrast, a general beta antibody which binds to the most
20 common and potent hCG antigenic site previously designated
by us as site II (B108 or B207) binds well simultaneously
with both B151 and B152 antibodies. B152 binds
simultaneously to all antibodies tested except for those to
the beta COOH-terminal region (CTP) (28) in contrast to B151
25 which binds well to CTP antibodies. B151 may represent a
newly revealed hCG epitope which only exists on nicked hCG
as reported in this manuscript.

Using B152 as capture and B207 or B108 as detection antibody,
30 produces an assay which measures all normal pregnancy forms
of hCG (both intact and nicked and beta subunit) to a similar
extent but prefers binding to the form of hCG or beta subunit
from its immunogen, C5. As predicted earlier from the liquid
phase studies, this assay does not prefer nicked forms of hCG
35 but hyperglycosylated forms of hCG such as C5. B152 and CTP
104 as well as several other monoclonal antibodies to the

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COOH-terminal region of HCG beta cannot bind simultaneously to C5, implying that this region is part of or very close to the epitope of B152. These data taken together with the apparent B152 preference for hyperglycosylated hCG, implies

5  that the carbohydrate of the CTP region may be part of the B152 epitope.   Further studies of the behavior of B152 in two-site assays confirm this hypothesis as detailed below.

Characteristics of the B152-B207-I$^{125}$ radioimmunometric two-
10 site assay

In order to better understand what this assay is measuring, we compared the relative binding potencies of a series of isoforms of hCG shown in figure 9 (also see methods):1. C5, choriocarcinoma hCG 2. 814, non-nicked hCG. 3. 813, nicked

15 hCG (80% nicked). 4. M4 mole-derived hCG, 98% nicked hCG with negligible hyperglycosylation. 5. MIA hCG, non-nicked and not significantly hyperglycosylated but missing 80% of the hCG beta COOH-terminus.   The B152 two-site assay prefers to bind to C5, its immunogen, but shows nearly equal recognition of

20 both  813  and  814,  nicked  and  non-nicked  hCG  of  normal pregnancy.    This  confirms  that  B152  does  not  display significant preference for the nicked form of hCG but rather for the form with carbohydrate differences.   This is also confirmed by the potency of M4 which is also 100% nicked as

25 is C5 but is not hyperglycosylated and displays a potency similar to CR127 hCG whether nicked or non-nicked.    M1A is the least potent ligand and is the only one missing most of its beta COOH-terminal peptide confirming the role of this region in the B152 epitope.

30 In order to further explore the nature of the B152 binding site, a commercially available peroxidase-labeled general hCGβ antibody (4001) was employed as a detection antibody in a two-site enzyme immunometric system.   Eight different hCG forms were evaluated in this system illustrated in Figure 14.

35 Results  are  analyzed  in  terms  of  relative  immunopotency (based on the slope of the regression line) in Figure 10.

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Linear regression correlation analysis was performed to compare the relationship of the immunopotencies of preparations 814, C5, M4, C7 and P8 one at a time with the carbohydrate differences as well as nicking differences among
5 the 5 heterodimeric isoforms of hCG. The correlation results for each comparison are as follows: 1. Tetrasaccharide O-linked core: $R^2=0.9147$  $P=0.0108$, significant; 2. Trianntennary branched moieties N-linked on $\beta$: $R=0.8853$ $P=0.0171$, significant; 3. Sialic acid O-linked: $R=0.3062$
10 $P=0.3332$, not significant; 4. Sialic acid N-linked on $\beta$: $R^2=0.2289$ $P=0.4149$, not significant; 5. Percent nicking in $\beta$ subunit: $R=0.0984$ $P=0.6072$, not significant.

To summarize this analysis, the immunopotency of hCG is
15 isoforms measured with B152 as a capture antibody in a two site assay correlates best with hyperglycosylated core moieties of both O and N-linked amino acids but not with sialic acid residues not with the degree of nicking of the $\beta$ subunit.

20

**Discussion**
The various isoforms of hCG have received increasing attention during recent years for their potential diagnostic value in problem pregnancies such as Down syndrome,
25 preeclampsia, trophoblastic diseases and early pregnancy loss. Nicked hCG at high concentrations has been associated with trophoblastic disease and other abnormal states of hCG production. Although nicked hCG has been measured by a variety of qualitative techniques such as immunoblotting, as
30 well as by direct isolation and sequence analysis of such hCG isoforms from urine, it is only recently that some investigators have been measuring nicked forms of hCG by a variety of subtractive immunoassays or non-specific hCG assays with the addition of scavenger antibodies since
35 direct, relatively specific antibodies were not available

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(22;29).   We had earlier shown that our commonly used
antibody, B109, to heterodimeric hCG reacted poorly to nicked
forms of hCG and this antibody was employed by other groups
in subtractive methods in attempts to quantify the content
5 of nicked hCG in blood and urine(22;29).


In this report, we described development of two antibodies
produced using choriocarcinoma-derived nicked hCG as
immunogen.  This led to a direct assay for nicked hCG.  By
10 employing a nicked, hyperglycosylated form of hCG from a
single choriocarcinoma patient, we have developed two
antibodies with distinct specificities.  One antibody (B151)
prefers binding to nicked forms of hCG, regardless of the
origin of the hCG molecule ( normal pregnancy or
15 choriocarcinoma).   Nick-free CR127 hCG has the lowest
affinity to this antibody as would be expected for a nick-
directed antibody.   While B151 was directed towards an
epitope dependent upon peptide bond cleavages in beta loop
2, B152 binding was not affected by peptide bond cleavages
20 within this loop.  Since the main difference between C5 hCG
and CR 127 hCGn was the hyperglycosylation of the former, it
was inferred that B152 was chiefly a carbohydrate-directed
antibody.   When B151 is used as capture antibody and
virtually any general beta antibody as detection antibody in
25 a two site assay, little cross-reaction with hLH is observed.
B151 cannot bind simultaneously with antibodies that are
directed chiefly to the hCG beta core region (site IV such
as B201 and B204) nor can the antibody bind with site III
antibodies directed to heterodimeric hCG such as B109 or A109
30 but it can bind at the same time as antibodies to the beta
COOH-region.  B151 may represent a new hCG epitope revealed
after nicking of beta loop 2.  The creation of new epitopes
by nicking of the hCG molecule has been reported by others
(30).

35

The second antibody, B152, preferentially binds hCG with the type of carbohydrate modifications prevalent in choriocarcinoma CG C5 regardless of the state of the nicking of the polypeptide chain. This was shown by the measurement

5 of the relative immunopotencies of several well characterized hCG isoforms as compared to their contents of N and O-linked carbohydrate moieties, sialic acid, and percentages of nicking. A significant correlation of B152 binding to hyperglycosylated hCG isoforms but not to those with sialic

10 acid or nicking differences was demonstrated. Antibody B152 appears to have at least partial specificity towards the hCG beta COOH-terminal region. This is shown by failure of a monoclonal antibody to bind simultaneously with beta COOH terminal antibodies such as CTP 104. In studies of B152 as

15 a two site assay, it was shown that hCG isoform MIA which is missing most of its COOH-terminal region binds poorly to B152.

Each antibody has a different application in accordance with

20 its specificity. Up to the present time, only indirect assays were available to quantify nicked hCG, such as subtractive assays or assays with scavenger antibodies added. Development of B151 permitted formulation of direct assays for nicked hCG which have been applied in studies of early

25 pregnancy (25,31). These measurements may have diagnostic applications for certain cancers (18-19) and in the detection of Down Syndrome (22). Application of the B152 antibody resulted in development of assays which can detect differences in the carbohydrate portion of hCG. Major

30 potential applications of this antibody include detection of Down Syndrome (23) and recognition of pregnancies destined for early pregnancy loss (25,32) This antibody is unusual since it is rare for carbohydrate discriminating antibodies to be developed to hCG. The only earlier such development

35 was antibodies to the hCG beta COOH-terminal region by the use of the isolated peptide bound to carrier (28,33). It is

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of interest that at least part of the epitope of B152 is also directed towards the beta COOH-terminal region. As reported earlier, the C5 choriocarcinoma form of hCG was the only such hCG displaying 100% hexasaccharide structure on its O-Serine

5 linked carbohydrate moieties in the beta COOH-terminal region. This may have resulted in production of this rare antibody. A second choriocarcinoma derived hCG isoform, C7, displayed 69% of this same O-linked core hexasaccharide structure and proved to be of similar potency as C5 with the

10 B152 epitope.

In conclusion, we have produced two novel monoclonal antibodies each with potential clinical utility: B151 which can be used to measure nicked forms of hCG with better

15 specificity than any antibody reported. B152 which is a unique antibody to a choriocarcinoma form of hCG and can discriminate hyperglycosylated from standard pregnancy hCG.

**Materials and Methods**

20 Hormones

Nick-free hCG (814) and nicked hCG (813) were prepared from pooled urine standard hCG (batch CR 127) by hydrophobic chromatography, as described previously (14). C8 hCG was purified from an individual with normal pregnancy, M1 and M4

25 hCG were both purified form individuals with gestational trophoblastic disease (hydatidiform mole), and C5 and C7 hCG form individual with malignant trophoblastic disease(choriocarcinoma), as described elsewhere (16,26). The N-terminal peptide sequences of the separated α-and β-

30 subunits of CR127; P8, M1, M4, C5 and C7 hCG and complete N- and O-linked oligosaccharide structures have recently been published (26). Two peaks were observed by Sephacryl S100 HR chromatography during purification of hCG preparation M1. The peak eluting in the position of standard hCG (M1), and

35 the peak eluting later (M1A) were purified separately. M1

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and M4 hCG were both purified from individuals with hydatidiform mole. They were isolated as described earlier (16).

5  Immunogens

Choriocarcinoma hCG designated C5 was isolated from a single patient as described earlier (16) and its complete carbohydrate analysis has recently appeared (26).

10  Immunization of Mice

Mice were immunized intraperitoneal with choriocarcinoma hCG preparation C5 diluted in saline (600 µg/mouse) and mixed 1:1 with Freund's adjuvant. After 3 consecutive immunizations (50 µg/mouse) at 3-4 week intervals, animals were retested
15  for 3 months and then given an intraperitoneal booster immunization (34). Ten days after the last booster, the sera from mice were tested for binding (in liquid phase radioimmunoassays) to both iodinated CR 127 hCG and to iodinated C5 hCG.

20

Liquid Phase Assays

Liquid phase assays were performed using a solution of 80µl 0.3M PBS with ).02% sodium azide, 50 µl tracer, 20µl normal mouse serum, 50µl 1% horse serum free of gamma globulin for
25  titrations ( when competition studies were performed, 50µl of competitor solution were substituted to generate dose/response or Scatchard curves), 100µl of diluted sera followed by incubation 1 hr at 37C and then overnight at 4C. Next day 100µl rabbit anti-mouse sera was added, incubated
30  10 min at 37C, then 1 hr at ambient, then centrifuged, supernatant aspirated, and pellets counted. The mouse whose antisera had the greatest discrimination between binding of radiolabeled C5 and radiolabeled CR 127 hCG was sacrificed and their spleen's were used for hybridoma production. The

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methods and materials used for the fusion work were described earlier (27;34).

Characterization of Antibodies

5 The cloned monoclonal antibodies, B151 and B152 were each studied in the liquid phase competitive radioimmunoassays using C5 as radiolabeled ligand and C5 and CR127 hCG as competitors. The method is as described under liquid phase assay method. Affinity constants were calculated by
10 Scatchard plots as described earlier (35-37).

Two Site Assays

Two site assay testing was conducted at Yale University (enzyme immunometric; and Columbia University
15 (radioimmunometric) as described earlier (24,37). Briefly, Immulon microtiter wells are coated with capture antibody, at a titer determined to provide the best combination of sensitivity and range. The plates were then washed and then blocked with 1% BSA in PBS. After a further wash standards,
20 clinical samples and controls were added to the coated wells (200µL/well). Plates are incubated, then samples are removed and plates washed. Labeled antibody (either radiolabeled or peroxidase labeled) is then added, and plates incubated further. The response variable was generated by a gamma
25 counter (Packard Instruments Cobra) or by absorption spectrophotometry as appropriate. A cubic spline curve is generated for standard values, and sample values read from this curve. Regression lines and all graphs were created using Sigmaplot 4.01 from SPSS software, Chicago, Ill.
30 Linear regression analysis of immunopotency) as compared to each of the carbohydrate differences and nicking differences of the hCG isoforms was accomplished in Instat 1998, GraphPad Software, Inc., San Diego California USA.

### Third Series of Experiments

The data plotted in Figures 15, 16 and 17 were derived from IVF patients who had collected first morning void urines daily, commencing with the day of embryo transfer. These
5 subjects had participated in the "donor egg" variant of IVF therapy; i.e., the recipients did not receive exogenous hCG.

A total of 22 cycles were evaluated. In 10 of these cycles, no hCG was detected, indicating that the blastocyst failed
10 to implant in the endometrium. There were five early pregnancy losses, in which a pregnancy was established but failed to carry to term. There were three normal pregnancies with multiple (2-3) fetuses and four singleton pregnancies.

15 The multiple fetus pregnancies produced higher quantities of hCG than did the singleton pregnancies, as would be expected. However, in both categories of successful pregnancies, there was clearly an inversion of the B152/B109 isoform ratio, while in the failing pregnancies, this ratio remains
20 elevated, indicating that the relative persistence of B152 hCG isoform and B109 isn't the predominate isoform.

Ongoing statistical analysis is particularly directed at determination of minimum constellation of determinations
25 necessary to develop a clinically useful discriminant function for the detection of problem pregnancies at a point where useful discriminant intervention (quite possibly the administration of exogenous hCG) would be effective.

30 Loss of patient #10 (Figure 17D) best exemplifies this; her pregnancy was not lost until after sample collection had ceased. And on the basis of hCG concentration, one could not predict a trouble pregnancy - but with the comparison of ratio with concentrations would predict the problem when
35 other indicators appear to be normal.

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**What is claimed is:**

1.      A method of predicting pregnancy outcome in a subject
        by determining the amount of an early pregnancy
5       associated molecular isoform of hCG in a sample
        comprising:
        (a)  contacting a sample with an antibody which
             specifically binds to the early pregnancy
             associated molecular isoform of hCG under
10           conditions permitting formation of a complex
             between the antibody and the early pregnancy
             associated molecular isoform of hCG;

        (b)  measuring the amount of complexes formed,
15           thereby determining the amount of the early
             pregnancy associated molecular isoform of hCG in
             the sample; and

        (c)  comparing the amount early pregnancy associated
20           molecular isoform of hCG in the sample
             determined in step (b) with either (i) the
             amount determined for temporally matched, normal
             pregnant subject(s) or (ii) the amount
             determined for non-pregnant subject(s), wherein
25           the relative absence of the early pregnancy
             associated molecular isoform of hCG in the
             sample indicates a negative outcome of pregnancy
             for the subject.

30 2.   A method of predicting the likelihood of a negative
        pregnancy outcome in a female subject comprising:
        (a)  contacting a sample from the subject with a
             capture antibody which specifically binds to an
             early pregnancy associated molecular isoform of
35           hCG under conditions permitting formation of a

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complex between the antibody and the early pregnancy associated molecular isoform of hCG;

(b)   contacting any complex formed in step (a) with a labelled detection antibody under conditions permitting binding to the complex the capture antibody and the hCG isoform;

(c)   measuring the amount of labeled detection antibody bound to the complex so as to thereby determine the amount of the early pregnancy associated molecular isoform of hCG in the sample; and

(d)   comparing the amount early pregnancy associated molecular isoform of hCG in the sample determined in step (b) with the amount determined for a normal pregnant subject, wherein the relative absence of the early pregnancy associated molecular isoform of hCG in the sample indicates a negative outcome of pregnancy for the subject.

3.   The method of claim 1, step (a) further comprising a second antibody which specifically binds to hCG without substantially cross-reacting with said antibody under conditions permitting formation of a complex between the antibody and the early pregnancy associated molecular isoform of hCG.

4.   The method of claim 3, wherein the capture antibody is B152.

5.   The method of claim 4, wherein the detection antibody is B207.

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6.      A method of predicting the likelihood of a negative
pregnancy outcome in a female subject comprising:

(a)   contacting a sample from the subject with a
capture antibody which specifically binds to an
5           early pregnancy associated molecular isoform of
hCG under conditions permitting formation of a
complex between the antibody and the early
pregnancy associated molecular isoform of hCG;

10           (b)   measuring the amount of complexes formed,
thereby determining the amount of the early
pregnancy associated molecular isoform of hCG in
the sample; and

15           (c)   comparing the amount measured in step (b) with
the amount determined by contacting the same
sample with a second capture antibody which
specifically binds to intact non-nicked hCG
without substantially cross-reacting with
20           said antibody under conditions permitting
formation of a complex between the antibody and
the early pregnancy associated molecular isoform
of hCG and a second antibody, wherein a high
ratio of amounts determined for said first
25           capture antibody relative to the second capture
antibody indicates a positive outcome of
pregnancy for the subject, a low ratio indicates
a negative outcome of pregnancy for the subject.

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7.      The method according to claim 5, wherein the second
capture antibody is B109 and the second antibody is
B108.

35 8.    The method of claim 1, step (c) comprising comparing
the amount early pregnancy associated molecular

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isoform of hCG in the sample determined in step (b) with either (i) the amount determined for temporally matched, normal pregnant subject(s) or (ii) the amount determined for non-pregnant subject(s), wherein amounts of the early pregnancy associated molecular isoform of hCG in the sample similar to amounts of early pregnancy associated molecular isoform of hCG in temporally matched pregnant samples indicates a positive outcome, amounts of early pregnancy associated molecular isoform of hCG in the sample similar to amounts of early pregnancy associated molecular isoform of hCG.

9.    The method of claim 1, wherein the sample is a urinary sample or a blood sample.

10.    The method of claim 1, wherein the sample is an aggregate sample taken from at least one day.

11.    The method of claim 1, wherein the antibody is labeled with a detectable marker.

12.    The method of claim 11, wherein the detectable marker is a radioactive isotope, enzyme, dye, magnetic bead, or biotin.

13.    The method of claim 12, wherein the radioactive isotope is $I^{125}$.

14.    A method of predicting pregnancy outcome in a subject by determining the amount of an early pregnancy associated molecular isoform of hCG in a sample comprising:

(a)    contacting a capturing antibody which specifically binds to the early pregnancy associated molecular isoform of hCG with a solid

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matrix under conditions permitting binding of
the antibody with the solid matrix;

(b) contacting the bound matrix with the sample
    under conditions permitting binding of the
    antigen present in the sample with the capturing
    antibody;

(c) separating the bound matrix and the sample;

(d) contacting the separated bound matrix with a
    detecting antibody which specifically binds to
    hCG under conditions permitting binding of
    antibody and antigen in the sample;

(e) measuring the amount of bound antibody on the
    bound matrix, thereby determining the amount of
    early pregnancy associated molecular isoform of
    hCG in the sample;

(f) comparing the amount early pregnancy associated
    molecular isoform of hCG in the sample
    determined in step (e) with either (i) the
    amount determined for temporally matched, normal
    pregnant subject(s) or (ii) the amount
    determined for non-pregnant subject(s), wherein
    amounts of the early pregnancy associated
    molecular isoform of hCG in the sample similar
    to amounts of early pregnancy associated
    molecular isoform of hCG in temporally matched
    pregnant samples indicates a positive outcome,
    amounts of early pregnancy associated molecular
    isoform of hCG in the sample similar to amounts
    of early pregnancy associated molecular isoform
    of hCG in the non-pregnant samples indicates a
    negative outcome of pregnancy for the subject.

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15.     The method of claim 14, further comprising:
        (a)  removing of the sample from the matrix; and

        (b)  washing the bound matrix with an appropriate
 5           buffer.

16.     The method of claim 14, wherein the capturing
        antibody is B152.

10  17.     The method of claim 14, wherein the detecting
        antibody is B207.

18.     The method of claim 14, step (a) further comprising
        a second capturing antibody which specifically binds
 15         to intact non-nicked hCG without substantially cross-
        reacting with said antibody under conditions
        permitting formation of a complex between the
        antibody and the early pregnancy associated molecular
        isoform of hCG.
20
19.     The method according to claim 18, wherein the second
        capturing antibody is B109.

20.     The method of claim 14, step (d) further comprising
 25         a second detecting antibody which specifically binds
        to hCG without substantially cross-reacting with
        said antibody under conditions permitting formation
        of a complex between the antibody and the early
        pregnancy associated molecular isoform of hCG.
30
21.     The method of claim 14, step (f) comprising comparing
        the amount of the early pregnancy associated
        molecular isoform of hCG determined in step (e) for
        said antibody with the amount determined in step (b)
 35         for the second antibody, wherein a high ratio of
        amounts determined for said antibody relative to the

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second antibody indicates a positive outcome of
pregnancy for the subject, a low ratio indicates a
negative outcome of pregnancy for the subject.

5  22.    The method of claim 14, wherein the sample is a
          urinary sample or a blood sample.

   23.    The method of claim 14, wherein the sample is an
          aggregate sample taken from at least one day.

10

   24.    The method of claim 14, wherein the antibody is
          labeled with a detectable marker.

   25.    The method of claim 24, wherein the detectable marker
15        is a radioactive isotope, enzyme, dye, magnetic bead,
          or biotin.

   26.    The method of claim 25, wherein the radioactive
          isotope is $I^{125}$.

20

   27.    A method for determining the amount of early
          pregnancy associated molecular isoforms of in a
          sample comprising:
          (a)  contacting the sample with an antibody which
25               specifically binds to an early pregnancy
                 associated molecular isoform of hCG under
                 conditions permitting formation of a complex
                 between the antibody and the early pregnancy
                 associated molecular isoform of hCG; and

30

          (b)  determining the amount of complexes formed
               thereby determining the amount of early
               pregnancy associated molecular isoform of hCG in
               the sample.

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28.    The method of claim 27, wherein the antibody
       specifically binds a region of the early

       pregnancy associated molecular isoform of hCG
5      comprising a carbohydrate moiety.

29.    The method of claim 27, wherein the antibody is
       produced by the hybridoma cell line accorded ATCC
       Accession No.HB-12467 .
10
30.    The method of claim 27, wherein the antibody is B152.

31.    A diagnostic kit for determining the amount of early
       pregnancy associated hCG is a sample comprising:
15     (a)    An antibody which specifically binds to an early
              pregnancy associated molecular isoform; and

       (b)    a solid matrix to which the antibody is bound;
              and
20
       (c)    reagents permitting the formation of a complex
              between the antibody and a sample.

32.    The diagnostic kit of claim 31, wherein the antibody
25     is B108 as detection antibody and B109 as capture
       antibody.

33.    The diagnostic kit of claim 31, wherein the antibody
       is B207 as detection antibody and B152 as capture
30     antibody.

34.    The diagnostic kit of claim 31, 32 or 33 further
       comprising control sample(s) normal pregnant
       sample(s).

35

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35.    The diagnostic kit of claim 31, 32, or 33 wherein the
antibody is labeled with a detectable marker.

36.    The  diagnostic  kit  of  claim  35,  wherein  the
detectable marker is a radioactive isotope, enzyme,
magnetic bead, dye or biotin.

37.    The  diagnostic  kit  of  claim  36,  wherein  the
radioactive isotope is $I^{125}$.

38.    An antibody which specifically binds to an early
pregnancy associated molecular isoform of human
chorionic gonadotropin.

39.    The antibody of claim 38, wherein the antibody
specifically binds to a region of the early pregnancy
associated molecular isoform of human chorionic
gonadotropin comprising both a carbohydrate moiety
and protein part.

40.    The monoclonal antibody of claim 38 designated B152.

41.    A hybridoma cell accorded ATCC Accession No.HB-12467,
producing the monoclonal antibody of claim 40.

42.    The early pregnancy associated isoform of hCG of
claim 1.

43.    The early pregnancy associated isoform of hCG
recognized by the monoclonal antibody of claim 40.

44.    A method for detecting non-trophoblast malignancy in
a sample comprising:
(a)  contacting  a  sample  with  an  antibody  which
specifically  binds  to  the  early  pregnancy

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associated molecular isoform of hCG under conditions permitting formation of a complex between the antibody and the early pregnancy associated molecular isoform of hCG;

5

(b) contacting the sample with a second detection antibody which specifically binds to intact non-nicked hCG without substantially cross-reacting with said antibody under conditions permitting

10 formation of a complex between the antibody and the early pregnancy associated molecular isoform of hCG,

(c) measuring the amount of complexes formed,

15 thereby determining the amount of the early pregnancy associated molecular isoform of hCG in the sample; and

(d) comparing the amount of early pregnancy

20 associated molecular isoform of hCG in the sample determined in step (b) with the amount of early pregnancy associated molecular isoform of hCG in the sample determined in step (c), wherein a positive detection of early pregnancy

25 associated molecular isoform detected in step (b) and a relative absence of the early pregnancy associated molecular isoform of hCG

30 detected in step (c) indicates the presence of non-trophoblast malignancy in the sample.

45. The method of claim 44, wherein the antibody is B152.

35 46. The method of claim 44, wherein the complex formed in step (a) is detected by a detection antibody.

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47.     The method of claim 46, wherein the detection
        antibody is B207.


48.     The method of claim 44, wherein the second antibody
5       is B109.


49.     The method of claim 44, wherein the complex formed in
        step (b) is detected by a detection antibody.


10 50.  The method of claim 49, wherein the detection
        antibody is B108.


51.     The method of claim 44, wherein the non-trophoblast
        malignancy is ovarian malignancy or prostate
15      malignancy or some other non-trophoblast malignancy.


52.     The method of claim 44, wherein the sample is a
        urinary sample or a blood sample.


20 53.  A method for detecting gestational trophoblast
        disease in a sample from a subject comprising:

        (a)  contacting a sample with an antibody which
             specifically binds to the early pregnancy
25           associated molecular isoform of hCG under
             conditions permitting formation of a complex
             between the antibody and the early pregnancy
             associated molecular isoform of hCG;
        (b)  contacting the sample with a second antibody
30           which specifically binds to intact non-nicked
             hCG without substantially cross-reacting with
             said antibody under conditions permitting
             formation of a complex between the antibody and
             the early pregnancy associated molecular isoform
35           of hCG;

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    (c)   measuring the amount of complexes formed, thereby determining the amount of the early pregnancy associated molecular isoform of hCG in the sample due to binding with the first antibody, and late pregnancy associated molecular isoform of hCG in the sample due to binding with the second antibody;

    (d)   determining the ratio of early pregnancy associated molecular isoform of hCG to late pregnancy associated molecular isoform of hCG in the subject; and

    (e)   comparing the ratio of early pregnancy associated molecular isoform of hCG to late pregnancy associated molecular isoform of hCG in the sample determined in step (c) over time, wherein a continuing high ratio of early pregnancy associated molecular isoform of hCG to late pregnancy associated molecular isoform of hCG in the sample determined in step (c) indicates the presence of gestational trophoblast disease in the subject.

54.    The method of claim 48, wherein the antibody is B152.

55.    The method of claim 48, wherein the second antibody is B109.

56.    The method of claim 48, wherein the gestational trophoblast disease is choriocarcinoma or hydatidiform mole.

57.    The method of claim 48, wherein the sample is a urinary sample or a blood sample.

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58.     An antibody which specifically binds to an early
        pregnancy associated molecular isoform of human
        chorionic gonadotropin.

5  59.   The antibody of claim 58, wherein the antibody
        specifically binds to a region of the early pregnancy
        associated molecular isoform of human chorionic
        gonadotropin comprising a carbohydrate moiety.

10 60.   The monoclonal antibody of claim 58 designated B152.

   61.   A hybridoma cell accorded ATCC Accession No. HB-
        12467, producing the monoclonal antibody of claim 60.

15

   62.   The antibody of claim 58 labeled with a detectable
        marker.

   63.   The antibody of claim 58, wherein the detectable
20      marker is a detection antibody.

   64.   The antibody of claim 58, wherein the antibody
        specifically binds to the nicked form of human
        gonadotropin.

25

   65.   The antibody of claim 58, wherein the antibody
        specifically binds an epitope dependent upon peptide
        bond cleavage in beta loop 2.

30 66.   The antibody of claim 58, wherein the antibody binds
        the human gonadotropin at the beta COOH-terminal
        region.

## FIG. 1

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## FIG. 2A



## FIG. 2B
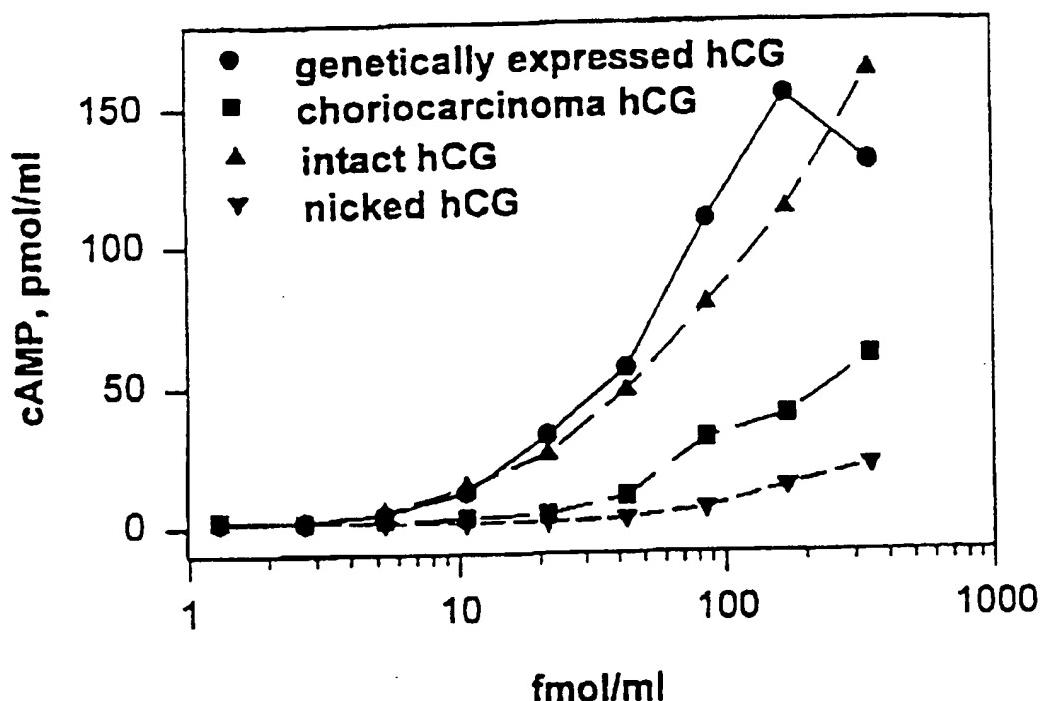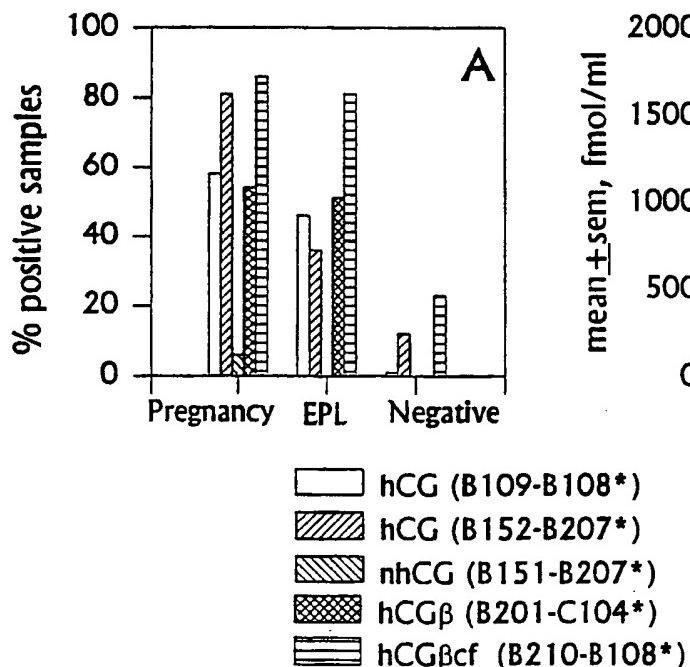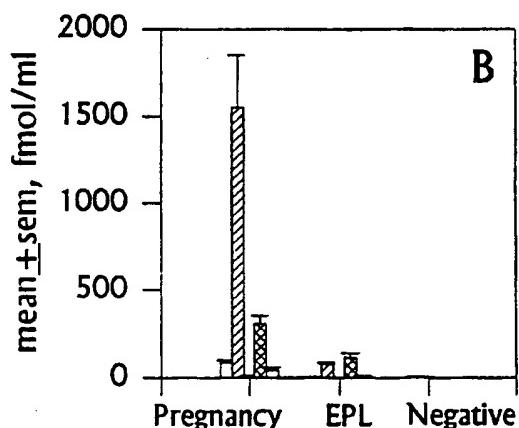


hCG (B109-B108*)
hCG (B152-B207*)
nhCG (B151-B207*)
hCGβ (B201-C104*)
hCGβcf (B210-B108*)

**FIG. 3A**
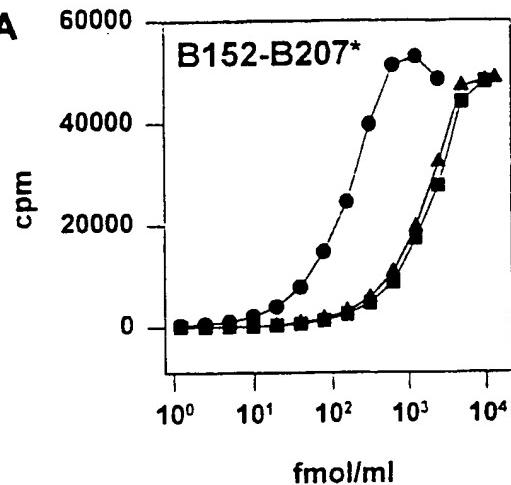


**FIG. 3B**
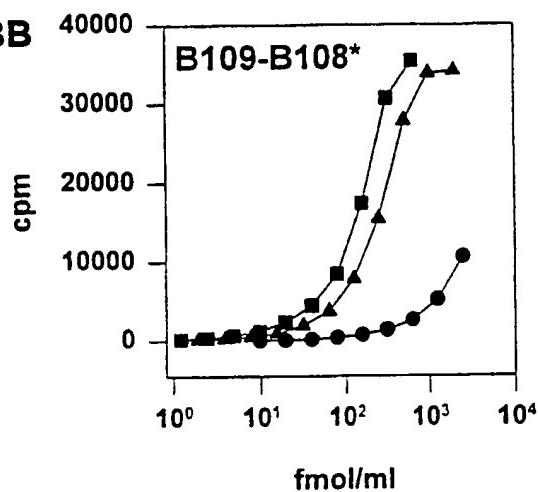


—●— choriocarcinoma hCG (C5)

—■— pregnancy hCG

—▲— pituitary hCG
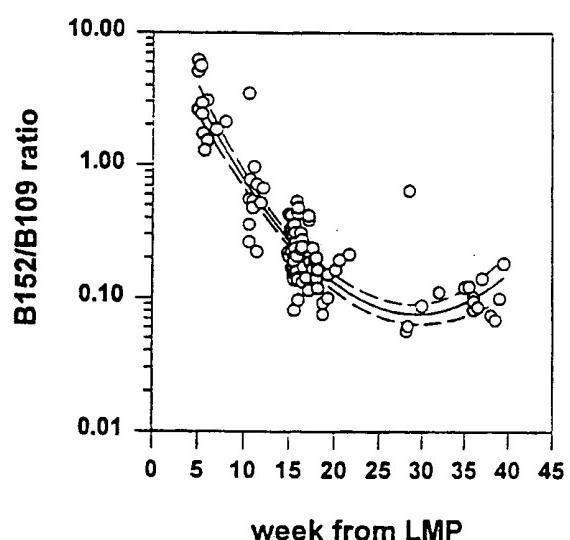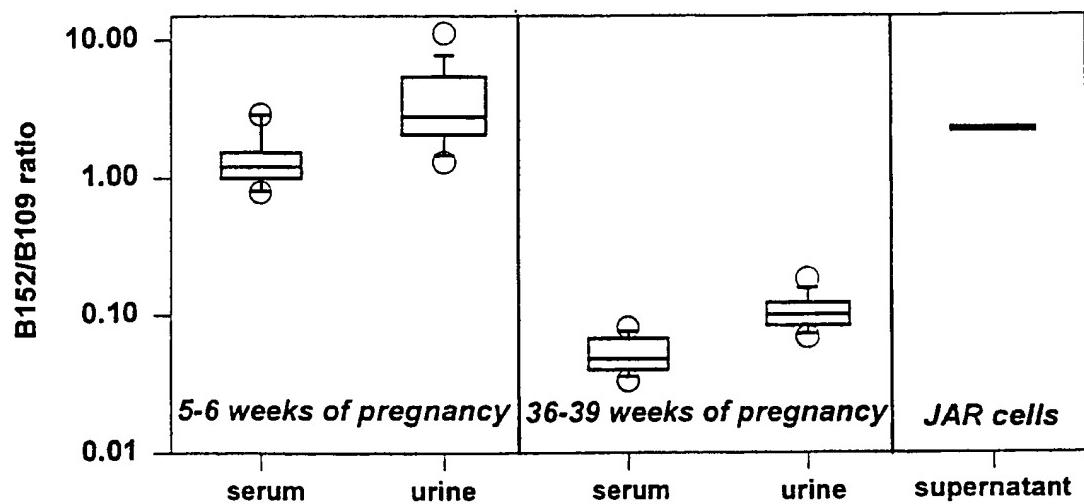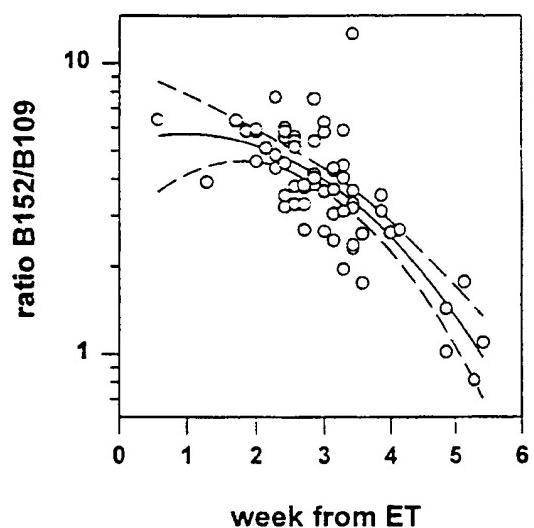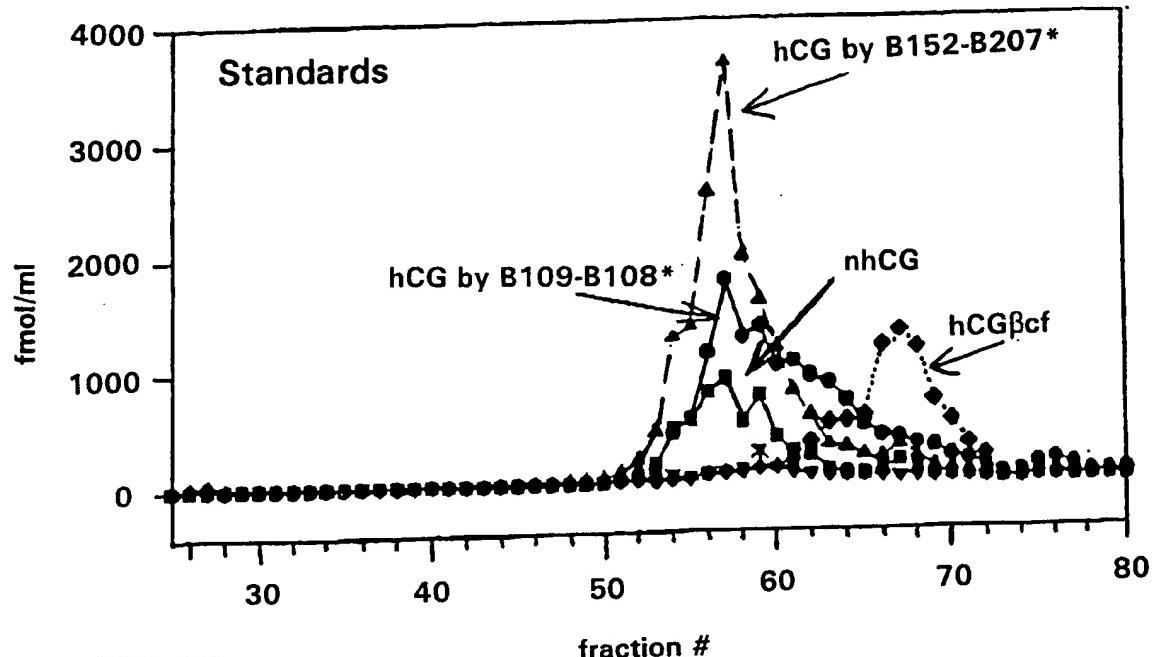
FIG. 4



week from LMP

**FIG. 5**

FIG. 6

FIG. 7A



FIG. 7B



● B-109-B108*    ▲ B152-B207*    ◆ B210-B108*

■ B151-B207*    ▼ B201-C104*

FIG. 8A

FIG. 8B

**FIG. 9A**



**FIG. 9B**



Dose (pmole/ml)

FIG. 10



Figure showing absorbance at 492 nm versus dose (pmole/ml). Legend: hCG 814 non-nicked, C5 chorioCG, hCGn M4, hCGn 813, C7 chorioCG, P8 pregnancy hCG, M4 hCGβ, C5 chorioCGβ. Annotation: "2 choriocarcinoma hCG preparations".
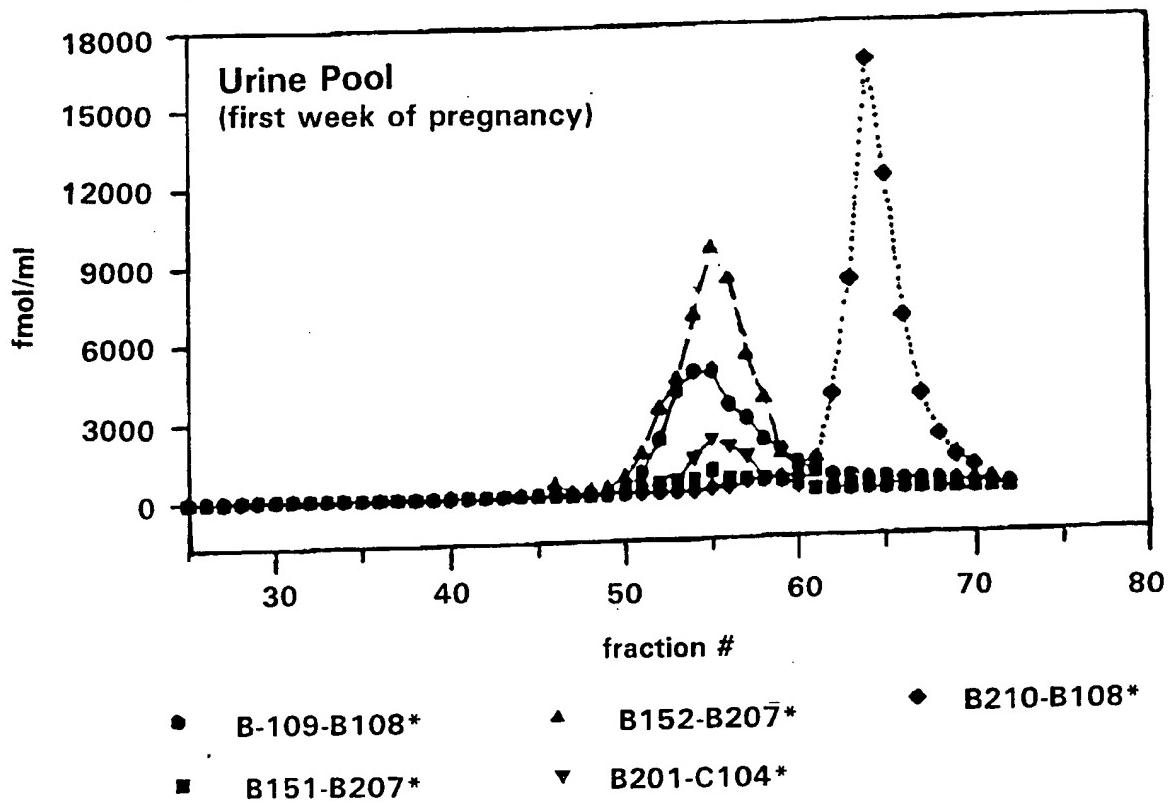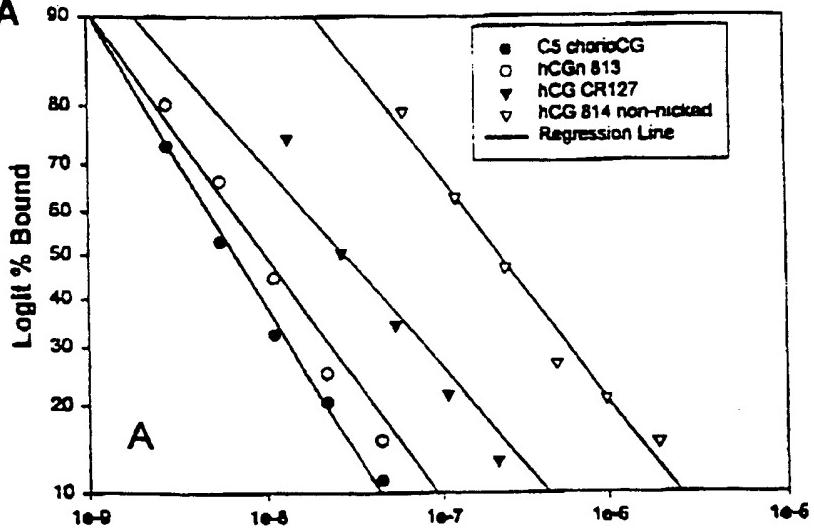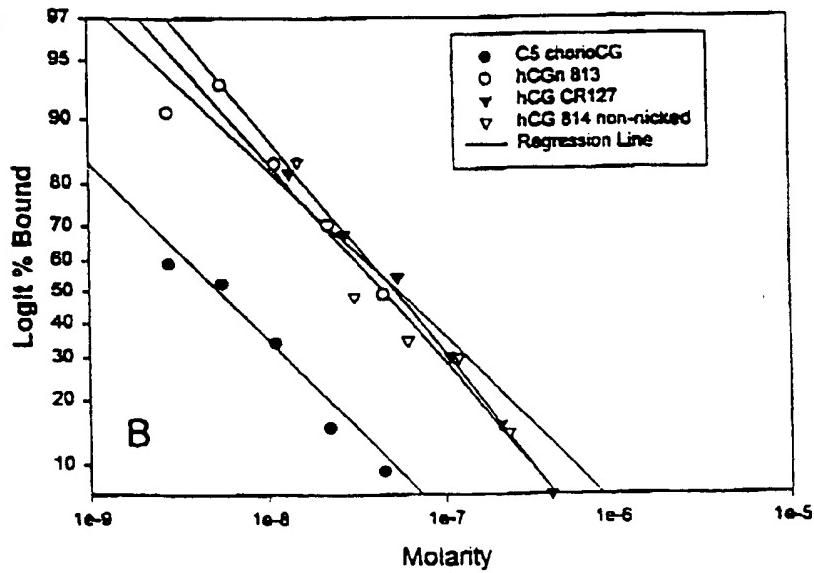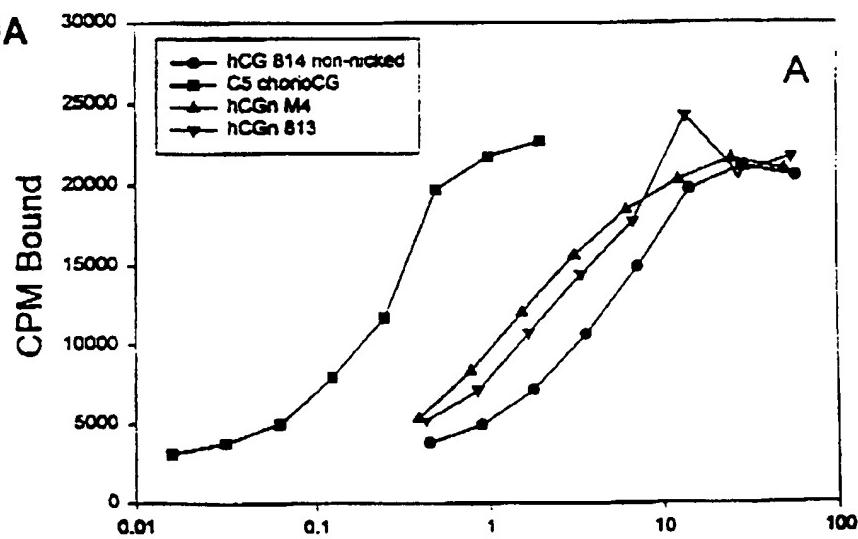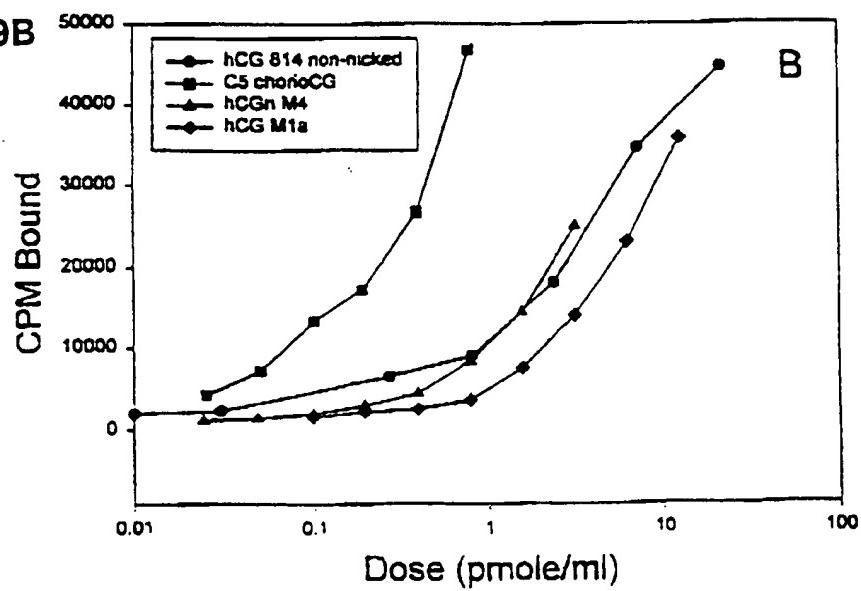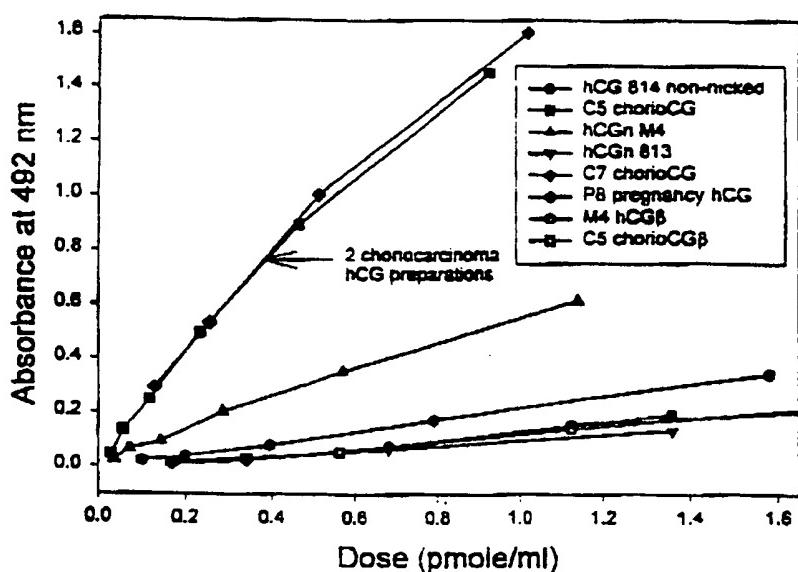
**FIG. 11**

Table I. Characteristics of the Reagents Used to Define Antibody Specificity. The peptide and carbohydrate structures of the reagents used were determined earlier (26). The % nicked β-subunit refers to the proportion of molecules with cleavages (missing peptide bonds) in the region β43 to β48. The % tetrasaccharide core is the proportion of O-linked oligosaccharides with tetrasaccharide (vs. disaccharide) core structure, and the % sialic acid, is the proportion of O-linked structures with antennae terminated by sialic acid residues. The proportion of triantennary N-linked oligosaccharides on β-subunit is given, as is the corresponding % sialic acid.

| Name | Source | N-Sialic acid[a] | O-Sialic acid[b] | % triantennary N-linked on β | % tetrasaccharide O-linked core | % β nicking |
|---|---|---|---|---|---|---|
| 814 hCG | CR 127 hCG[b] | 95 | 66 | 5 | 19 | 19 |
| C5 chorioCG | Choriocarcinoma | 95 | 58 | 48 | 100 | 100 |
| M4 mole CG | Mole pregnancy | 120 | 49 | 30 | 20 | 98 |
| 813 hCGn | CR 127 hCG | ND | ND | ND | ND[d] | 80 |
| C7 chorioCG | Choriocarcinoma | 68 | 53 | 48 | 69 | 3 |
| P8 hCG | Pregnancy | 94 | 73 | 21 | 13 | 0 |
| M4 mole β | Mole pregnancy | 120 | 49 | 30 | 20 | 98 |
| C5 chorio β | C5 chorioCG | 95 | 58 | 48 | 100 | 100 |
| CR 129 β | CR 129 hCG | 96 | 63 | 11 | 17 | 19 |
| HLH I-1 | Pituitary hLH | ND | NA[d] | NA | NA | NA |
| M1A | Mole pregnancy | 98.5 | NA | 16.5 | <15% CTP[e] | 24 |

[a] % sialic acid residues per sugar chain, N-linked on β.

[b] % sialic acid residues per sugar chain, O-linked on β.

[c] The "CR" series of hCG reference preparations were made at Columbia University and were distributed internationally as reference materials for purified hCG. CR 119 is also known as the 3rd international immunoassay reference preparation for hCG.

[d] ND is not done; NA is not applicable to that reagent.

[e] Less than 15% of the beta COOH-terminal region is present on this preparation.

**FIG. 12**

Table II: Affinity Constants[a] Determined by Liquid Phase Competition Assays Using C5 as Tracer Ligand

### Competitors

| Antibody | C5 chorioCG | Nicked hCG CR 127 (813) | Parent CR 127 hCG[b] | Nick-free hCG C R127 (814) |
|---|---|---|---|---|
| B151 | $4.4 \times 10^8$ | $3.8 \times 10^8$ | $4.2 \times 10^7$ | $1.3 \times 10^7$ |
| B152 | $3.5 \times 10^8$ | $5.4 \times 10^7$ | $4.7 \times 10^7$ | $5 \times 10^7$ |

[a] Ka as L/M

[b] hCG CR 127 is an NIH-distributed hCG reference preparation produced at Columbia University.

## FIG. 13A

Table III: Matrices of data for binding characteristics of different pairs of detection antibodies using B151 or B152 as capture antibody.

A. Relative Cross-Reactivities of Two Site Assay Using B151 as Capture Antibody

| Ligand | B207[a,c] | B204[a] | B201[a] | B108[a] | B109[a] | A109[a] | CTP104 |
|---|---|---|---|---|---|---|---|
| C5 | 100% | ✓[b] | ✓ | 100% | ✓ | ✓ | 100 |
| 813 CR 127 hCGn | 100% | ✓ | ✓ | 100% | ✓ | ✓ | 47% |
| 814 CR127 hCG | 12% | ✓ | ✓ | 37% | ✓ | ✓ | 14% |
| HCGβ | 2% | ✓ | ✓ | 2% | ✓ | ✓ | ✓ |
| C5β | 5% | ✓ | ✓ | | ✓ | ✓ | ✓ |
| HCGβ core | ✓ | ✓ | ✓ | | ✓ | ✓ | ✓ |
| HLH | 2% | ✓ | ✓ | 3% | ✓ | ✓ | ✓ |
| HLHβ | 5% | ✓ | ✓ | | ✓ | ✓ | ✓ |
| HCGα | ✓ | ✓ | ✓ | 3% | ✓ | ✓ | ✓ |
| Maximum binding[b] | 50% | 0% | 0% | 13% | 0% | 0% | 83% |

[a] labeled detection antibodies
[b,c] out of low range detection
[c] this particular assay format was applied in O'Connor et al (25).

## FIG. 13B

B. Relative Cross-Reactivities of Two Site Assay Using B152 as Capture Antibody

| Ligand | B207[d] | B204[a] | B201[a] | B108[a] | B109[a] | A109[a] | CTP104[a] |
|---|---|---|---|---|---|---|---|
| C5 | 100% | 100% | 94% | 42% | 53% | 100% | ∨ |
| 813 CR 127 hCGn | 10% | ∨[c] | ∨ | 15% | 32% | 64% | ∨ |
| 814 CR127 hCG | 7% | ∨ | ∨ | 30% | 100% | 26% | ∨ |
| HCGβ | 6% | 20% | 19% | 11% | ∨ | ∨ | ∨ |
| C5 β | 190% | 100% | 100% | 100% | ∨ | ∨ | ∨ |
| HCGβ core | <1% | ∨ | ∨ | ∨ | ∨ | ∨ | ∨ |
| HLH | <1% | ∨ | ∨ | ∨ | ∨ | ∨ | ∨ |
| hLHβ | <1% | ∨ | ∨ | ∨ | ∨ | ∨ | ∨ |
| HCG α | <1% | ∨ | ∨ | ∨ | ∨ | ∨ | ∨ |
| Maximum binding[b] | 64% | 2% | 44% | 80% | 2% | 14% | 25% |

The molar quantity of ligand required to produce binding equal to 50% of the maximum binding achieved by C5 was determined. Cross-reactivity shown in this table as a percentage is calculated by dividing the molar quantity of the standard by the molar quantity of the other ligand at 50% maximum binding dose.

[a] labeled detection antibodies

[b] maximum binding represents the total quantity of radiolabeled detection antibody which can bind to the plate in the system described.

[c] < out of low range detection

[d] this particular assay format was applied in O'Connor et al (25).

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FIG. 14

Table IV. Immunoreactivity of antigens in the B152 immunoradiometric assay. The dose-response curves used to provide data for this table are shown in Figure 3. Each curve was fitted with 4-5 points. Slope and coefficient of determination ($R^2$) were determined using a non-linear regression algorithm. Slopes were used as an indicator of antigen potency. Relative potency was estimated as the slope of antigens relative to the slope of C5 Choriocarcinoma hCG (the immunogen).

| Reagent | Slope [a] | S.E. | $R^2$ | Relative Potency |
|---|---|---|---|---|
| 814 hCG | 0.1588 | 0.0098 | 0.992 | 10.2% |
| C5 chorioCG | 1.5603 | 0.1015 | 0.983 | 100% |
| M4 mole hCGn | 0.5317 | 0.0240 | 0.992 | 34% |
| 813 hCGn | 0.0986 | 0.0021 | 0.999 | 6.3% |
| C7 chorioCG | 1.4515 | 0.1246 | 0.985 | 93.0% |
| P8 hCG | 0.2192 | 0.0031 | 0.999 | 14.0% |
| M4 mole hCGβ | 0.1038 | 0.0069 | 0.991 | 6.67% |
| C5 chorioCGβ | 0.1286 | 0.0042 | 0.998 | 8.24% |
| CR 129 hCGβ | Only 2 points | | | |
| HLH batch I-1 | No response | | | |

[a] Slope are from figure 3 as calculated in Sigmaplot 4.01 by linear regression analysis. Units of slope are pmole/ml absorbance at 492nm.

Figure 15

Figure 16

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## Figure 17



**A** Patient 1
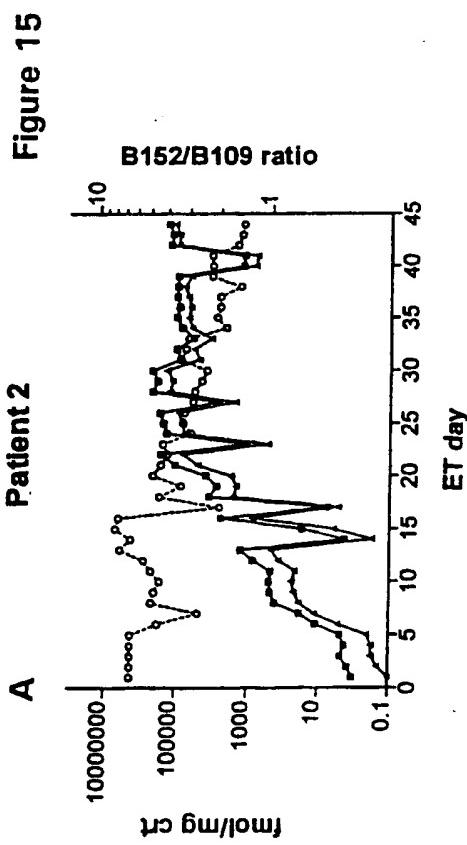
**B** Patient 5
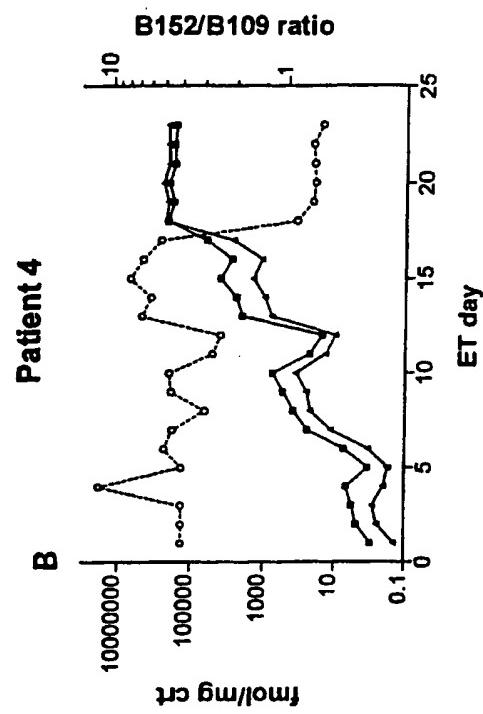
**C** Patient 7
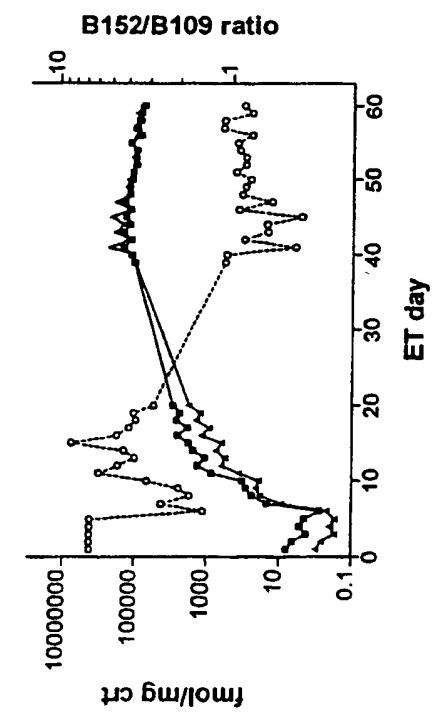
**D** Patient 10

**E** Patient 11
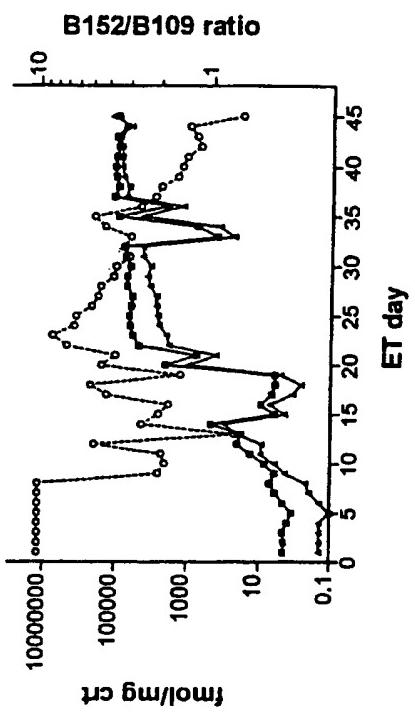
—●— hCG as determined by assay B152-B207*, fmol/mg creatinine

—▲— hCG as determined by assay B109-B108*, fmol/mg creatinine

--○-- Ratio=B152-B207*/B109-B108*
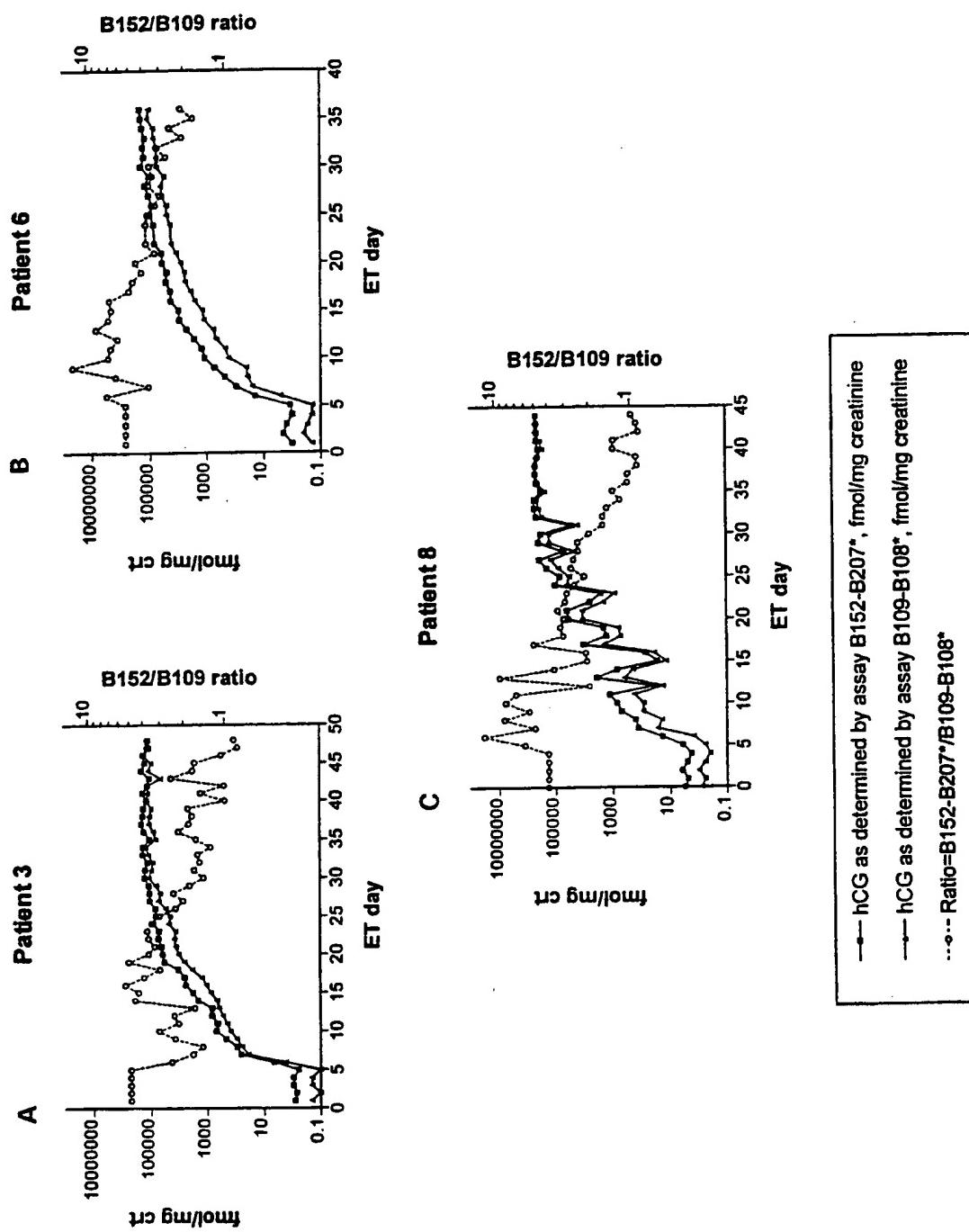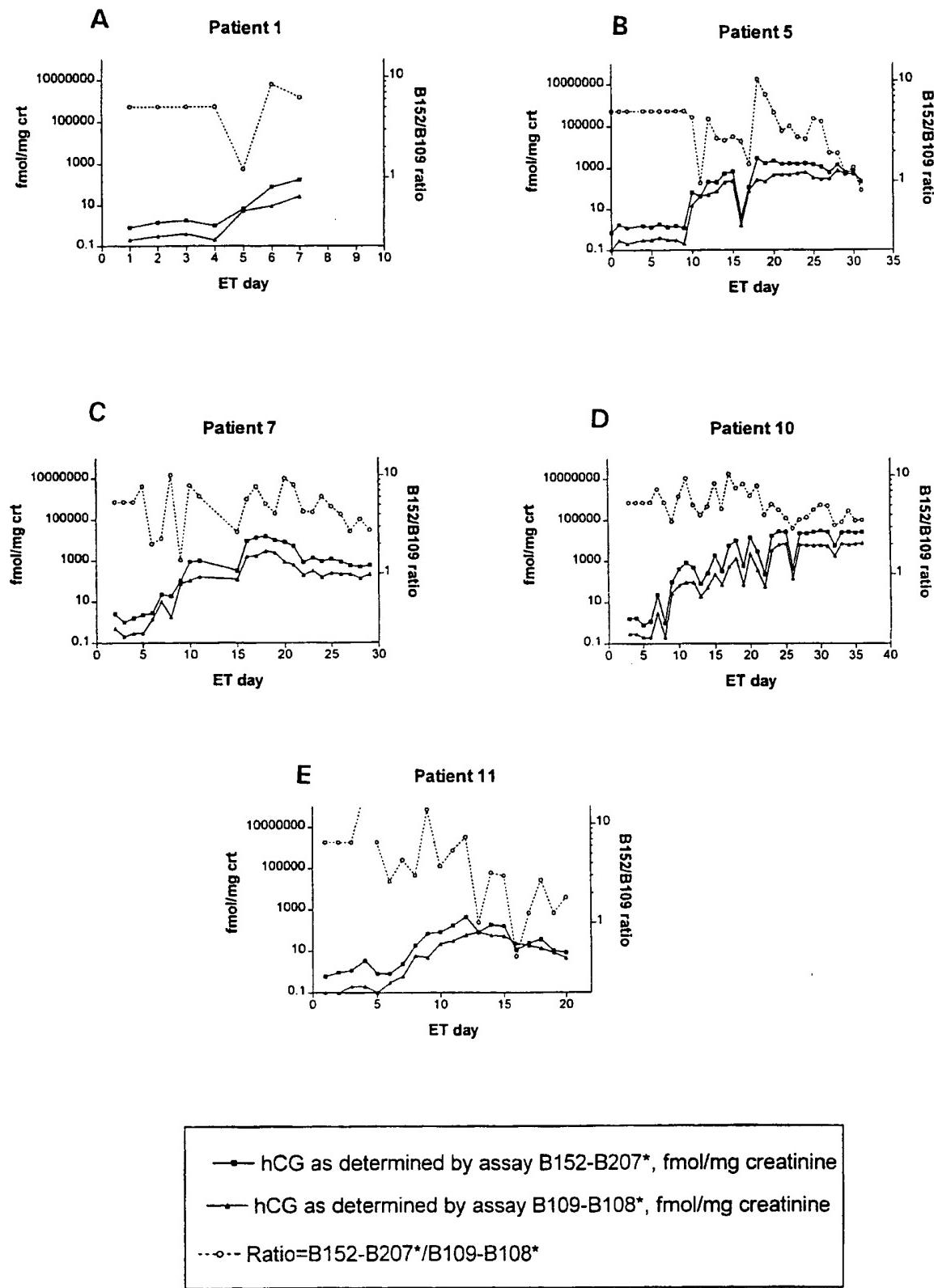
**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(7) :Please See Extra Sheet.

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EAST and WEST Patent Database, STN-biosis, caplus, haplus, medline.

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
| --- | --- | --- |
| X,P | WO 99/41584 A2 (THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK) 19 August 1999, see full document. | 1-66 |
| X | BIRKEN, S. et al. Development and Characterization of Antibodies to a Nicked and Hyperglycosylated Form of hCG from a Choriocarcinoma Patient. Endocrine. April 1999, Vol. 10, No. 2, pages 137-144, see abstract. | 1-66 |
| Y | KOVALEVSKAYA, G. et al. Evaluation of Nicked Human Chorionic Gonadotropin Content in Clinical Specimens by a Specific Immunometric Assay. Clinical Chemistry. January 1999, Vol. 45, No. 1, pages 68-77, see abstract. | 1-66 |

| ☒ Further documents are listed in the continuation of Box C. | ☐ See patent family annex. |
| --- | --- |

| * | Special categories of cited documents: | "T" | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| --- | --- | --- | --- |
| "A" | document defining the general state of the art which is not considered to be of particular relevance | | |
| "B" | earlier document published on or after the international filing date | "X" | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| "L" | document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | "Y" | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| "O" | document referring to an oral disclosure, use, exhibition or other means | | |
| "P" | document published prior to the international filing date but later than the priority date claimed | "&" | document member of the same patent family |

| Date of the actual completion of the international search | Date of mailing of the international search report |
| --- | --- |
| 21 JUNE 2000 | 11 AUG 2000 |

| Name and mailing address of the ISA/US | Authorized officer |
| --- | --- |
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**C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | COLE, L. A. et al. The Heterogeneity of Human Chorionic Gonadotropin (hCG). III. The Occurrence and Biological and Immunological Activities of Nicked hCG. Endocrinology. 1991, Vol. 129, No. 3, pages 1559-1567, see full document. | 1-37, 42, and 44-57 |
| Y | BERGER, P. et al. Variants of Human Chorionic Gonadotropin from Pregnant Women and Tumor Patients recognized by Monoclonal Antibodies. Journal of Clinical Endocrinology and Metabolism. 1993, Vol. 77, No. 2, pages 347-351, see full document. | 1-66 |
| Y | ACEVEDO, H.F. et al. Expression of Membrane-associated Human Chorionic Gonadotropin, Its Subunits, and Fragments by Cultured Human Cancer Cells. Cancer. 01 April 1992, Vol. 69, No. 7, pages 1829-1842, see full document. | 1-66 |
| A | HOERMANN R. et al. Molecular Heterogeneity of Human Chorionic Gonadotropin in Serum and Urine From Patients with Trophoblastic Tumors. Clinical Investigation. 1993, Vol. 71, pages 953-960, especially pages 958-959. | 1-66 |

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**A. CLASSIFICATION OF SUBJECT MATTER:**
IPC (7):

C12Q 1/68, 1/70; G01N 21/64, 33/00, 33/53, 33/531, 33/533, 33/543; C07H 21/02, 21/04; C12N 15/00; B05D 1/18; B01J 13/00

**A. CLASSIFICATION OF SUBJECT MATTER:**
US CL :

435/5, 6, 7, 7.21, 7.9, 91.1, 91.2, 94, 183, 270; 436/501, 504, 508, 518, 808, 811; 536/23.1, 24.3, 24.31, 24.32, 25.32; 935/1, 2, 3, 5, 8, 19, 76, 77, 78, 85, 111; 530/300, 350, 387.1, 388.1, 388.2, 391.1, 811, 812, 815;

**B. FIELDS SEARCHED**
Minimum documentation searched
Classification System: U.S.

435/5, 6, 7, 7.21, 7.9, 91.1, 91.2, 94, 183, 270; 436/501, 504, 508, 518, 808, 811; 536/23.1, 24.3, 24.31, 24.32, 25.32; 935/1, 2, 3, 5, 8, 19, 76, 77, 78, 85, 111; 530/300, 350, 387.1, 388.1, 388.2, 391.1, 811, 812, 815;

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